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THE CHROMOSOMES OF PRIMARY MAMMARY CARCINOMAS IN MILK VIRUS STRAINS OF THE MOUSE

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I. INTRODUCTION

DATA on the chromosome conditions of malignant tumours published by various authors demonstrate that the chromosomes in malignant tumour cells very often behave differently from those of normal tissue. However, previous studies on tumour chromosomes have dealt mainly with tumours in the ascites form. The reason for this specialization of the studies is that ascites tumours were found to be much more favourable for chromosome analysis than the solid type of tumour. But few authors have published data on solid tumour chromosomes (SACHS and GALLILY, 1956; FRITZ-NIGGLI, 1956). It is, however, very important to know the chromosome constitution of the primary tumour also in order to understand the changes that take place during the development of the tumours and during serial transplantations.

In order to contribute to this question a study was started on the chromosome conditions of primary mammary adenocarcinomas arising spontaneously in dba and C3H mice. These two inbred strains are characterized by a high incidence of spontaneous tumours in the mammary glands. These strains are known to contain a virus or virus-like factor, however, that is of essential importance for the development of their breast tumours. The chromosome conditions found by us in these tumours were in certain respects of a rather special kind. This makes it reasonable to suppose that their virus-tumour nature has been of importance in this connection and that for this reason our tumours cannot be considered as representative instances of ordinary spontaneous primary tumours.

In all 19 tumours were studied. They were collected from 11 dba and 7 C3H females. One of the dba mice had developed two tumours

TABLE 1. *Chromosome numbers of primary mammary adenocarcinomas in mice.*

Tumour No.	Mouse strain	Age in months of animal when sacrificed	No. of cells analyzed	Percentage of cells with the chromosome numbers						
				39	40	41	78	79	80	81
1	dba	12	169	2	95	3				
2	dba	10	167	4	90	6				
3	dba	12	150	2	90	6			2	
4	dba	11	131	1	95	3			1	
5	dba	11	159	2	89	4			5	
6	dba	13	109	2	96	1			1	
7	dba	14	139	2	95	2			1	
8	dba	9	125	1	97	1			1	
9	dba	9	144	1	98	1				
10	dba	6	121		99	1				
11	dba	4	108	1	97	2				
12	dba	10	145		58				42	
13	C3H	11	162	4	95	1				
14	C3H	7	188	4	91	4			1	
15	C3H	7	120		100					
16	C3H	8	134	100						
17	C3H	10	122		99	1				
18	C3H	8	150	2	94	2			2	
19	C3H	7	120				3	3	92	2

simultaneously. The animals were 4 to 13 months old when sacrificed. The chromosome studies were made in acetic-orcein preparations. The animals were given a pretreatment with colchicine (intraperitoneal injection of 60 gamma per 10 grams of animal) for one hour, sacrificed and a piece of tumour tissue kept for 15—30 min. in a hypotonic solution (1.1 %) of sodium citrate. Acetic orcein preparations were then made in the ordinary way. The method of using a pretreatment with hypotonic sodium citrate was introduced by FORD and HAMERTON (1956).

II. OBSERVATIONS

The chromosome numbers of the tumours studied are given in Table 1. Only reliable metaphase cells in which exact counts could be made were taken into consideration. As can be seen from the table the numerical chromosome variation in this material is very narrow. In 16

of the tumours cells with 40 chromosomes are predominant, the frequency of cells with this number being from 89 to 100 %. In one tumour all of the cells had 39 chromosomes (134 cells were counted). In another tumour 58 % of the cells had 40 chromosomes and 42 % of the cells had 80 chromosomes. Furthermore, one tumour was entirely tetraploid, 92 % of the cells showing 80 chromosomes and the remaining 8 % showing 78, 79 and 81. No structural changes of the chromosomes could be recognized with regard to such properties as chromosome size, position of centromeres and the occurrence of secondary constrictions. A chromosome change of another and more special kind was found, however, in our tumours. This will be described further below.

Even though the variation in chromosome number observed in this tumour material is small, it is, however, distinctly greater than that occurring in normal tissue. We have studied the chromosomes of regenerating liver in adult mice as well as of liver and spleen in newly born mice. All diploid cells of these tissues were found to have exactly 40 chromosomes. The only deviations observed were due to polyploidy. About 150 diploid cells were analyzed in each organ.

On the other hand, the primary tumours studied here differed even more in their chromosomal conditions from the old established mouse tumours studied earlier by various investigators (*e. g.* BAYREUTHER, 1952; LEVAN and HAUSCHKA, 1952; TJIO and LEVAN, 1954). These tumours were of the ascites type and had been maintained for several years by means of serial transplantations. Their chromosome conditions often differed greatly from normal tissue both with regard to the number and the structure of the chromosomes. It is important to find out when and how these chromosomal deviations arise during the evolution of the tumours. For this reason we have initiated a study of the development of the chromosome conditions in four of the spontaneous mammary tumours during serial transplantations. Up to the present we have followed four transplant generations and no gross chromosomal changes have been found.

As has already been mentioned no chromosome structural changes of the ordinary kind were found in the present tumour material. Another kind of chromosomal change of a more surprising and unusual type, however, was found in practically all of the tumours studied. The only exception is tumour no. 16, which differed from the other tumours also in that its chromosome number was 39 (all the other tumours having either 40 or 80 as stemline numbers). The abnormality in question consists in the appearance of a special kind of heteropycnotic chromosome.

All the diploid tumours, with the exception of no. 16, turned out to have a single chromosome of this heteropycnotic type (Figs. 1—6). The tetraploid tumour, no. 19, had two heteropycnotic chromosomes and the mixoploid tumour, no. 12, showed one chromosome of this kind in its diploid cells and two in its tetraploid cells. This chromosome type deviates from the normal type in that it shows a positive heteropycnosis, which is especially clear during prophase, telophase and the transition stage between metaphase and telophase. Because of the colchicine treatment these cells do not pass a typical anaphase, but there is a direct transition from metaphase to a telophase stage with continued association of the chromatids to one another in (or close to) the centromeres, as observed at c-mitosis in ascites cells by LEVAN (1954). The heteropycnotic chromosome in the present material is also sometimes faintly heteropycnotic at full metaphase. The chromosome in question also forms a chromocentre in the resting nuclei, but these are often more difficult to analyse in this respect because of the presence of other chromocentres.

The chromosome conditions in tumour no. 16, which had 39 chromosomes and which lacked the heteropycnotic type, are shown in Figs. 7—10.

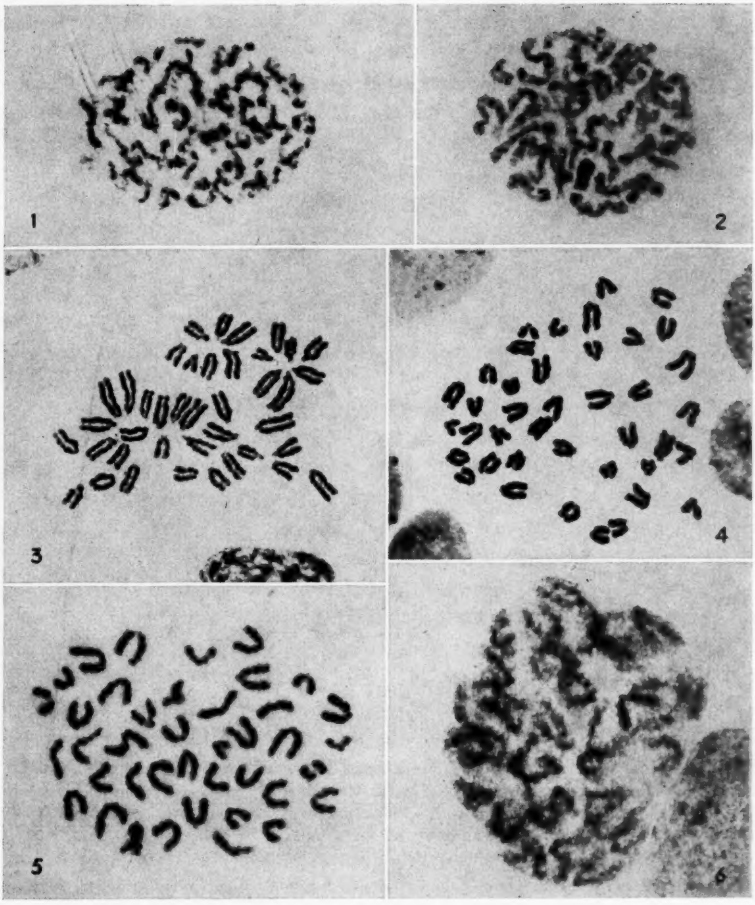
A thorough investigation of normal cells (liver and spleen) from female mice of these inbred strains failed to reveal any heteropycnotic chromosomes.

It would have been of great interest to establish whether the heteropycnosis observed here is manifested only in a special chromosome pair, or if more than one pair is capable of behaving in this way. Unfortunately, the chromosome morphology does not give much information as the chromosomes of the mouse are all telocentric and as the transition in size from the largest to the smallest pair is quite gradual. The observations, however, are in good agreement with the interpretation that it may be the same chromosome pair that shows a heteropycnotic member in all the cases studied. But this has by no means been proved.

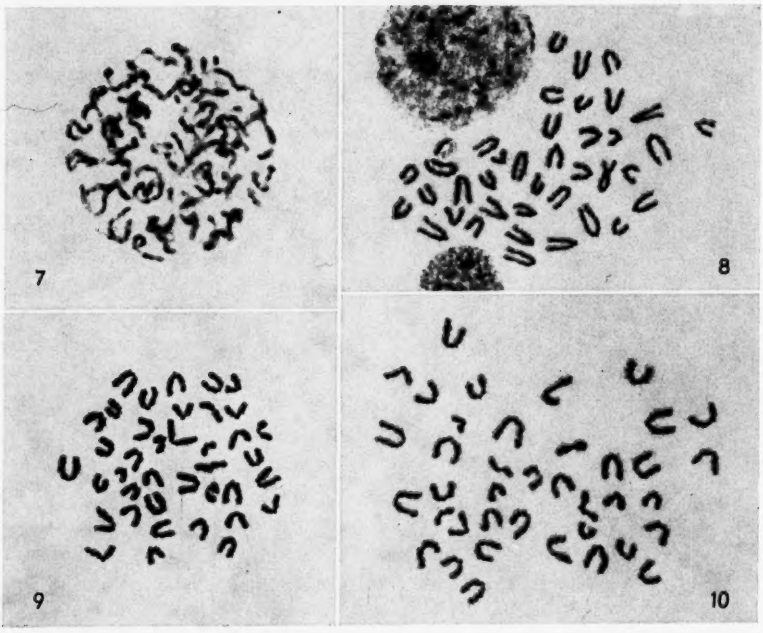
III. DISCUSSION

1. Introduction

The spontaneous mammary tumours studied here were found to be much less variable with respect to both chromosome number and karyotype than the old established ascites tumours studied earlier by various authors.



Figs. 1—6. Mitotic stages in spontaneous mammary tumours of mice having a stemline with 40 chromosomes. — Figs. 1—2. Prophase. Fig. 3. Prometaphase — early metaphase. Fig. 4. Full metaphase. Fig. 5. Transition stage between metaphase and telophase. Fig. 6. Late telophase. Note the heteropycnotic chromosome that is distinctly seen at all these stages except full metaphase. — ×900.



Figs. 7—10. Mitotic stages in a spontaneous mammary tumour of mice having a stemline with 39 chromosomes. — Fig. 7. Prophase. Figs. 8—10. Transition stages between metaphase and telophase. No heteropycnotic chromosome was found in this tumour. — ×750.

One reason that might be suggested to explain the comparatively normal chromosome conditions in the present tumours is that they were studied as primary tumours — that is, a relatively short period after they had arisen spontaneously. It is obvious that when a tumour has been propagated for a long time, there will be a gradual accumulation of various kinds of chromosomal changes, some of which may have contributed to an increased efficiency of the stemline of the tumour.

Other reasons for the difference between these mammary tumours and the ascites tumours are also conceivable. Thus, it is probable that malignant tumours may be heterogeneous with regard to their origin. In some cases they may originate through a *very specific change* of some kind (which may or may not involve the genotype) and in others possibly as the result of a more *general unbalance* of the genotype.

In this connection it is necessary to take into account the fact that the mouse strains used (dba and C3H) contain the famous so-called milk-factor discovered by BITTNER(*cf.* the review by HESTON, 1948). This is a virus or virus-like agent which occurs in certain strains of inbred mice and which is regularly transmitted from mother to progeny by means of the milk. This factor plays an essential role in the development of breast cancer in these mouse strains (DMOCHOWSKI, 1953; OBERLING and GUÉRIN, 1954). It is quite possible that the absence of the usual types of chromosome structural changes in our tumours may be causally connected in one way or another with the fact that these tumours have been provoked by a cancer virus.

Many carcinogenic agents are known to be very effective in provoking mutations and chromosome breakage, *e. g.* ionizing radiations and many mutagenic chemicals. It is probable that the carcinogenic effect of such agents may be a consequence of their ability to cause a general derangement of the genotype; some of the new cell genotypes that result may have acquired malignant properties. The absence (or rareness) of ordinary changes in the chromosome morphology of the tumour cells studied here suggests that the action of the milk factor is of another kind. This virus does not provoke a general increase in the frequency of mutations and chromosome breaks which occasionally give rise to malignant genotypes, but it induces malignancy in a more specific way, which is at present unknown. In any case, the observation of special heteropycnotic chromosomes in our tumours suggests that the chromosomes are involved in the origin of malignancy also in the case of the milk factor.

Some interpretations or hypotheses concerning the way in which the

milk factor may act in producing chromosomal heteropycnosis and malignancy are presented below.

2. The existence of a connection between heteropycnosis and malignancy

The heteropycnotic chromosomes found in the cells of the mammary carcinomas are remarkable particularly in two respects. The first point of interest is that they are present in the tumour cells but not in the normal tissue. The second surprising feature is that there is only a single chromosome of this type in each cell. If they had been observed in males, it would have been natural to suggest that they were either the Y or the X chromosomes, as these chromosomes are represented only once per cell in the males. It is more difficult to understand the present findings in which we are concerned with female cells. Each individual should have two identical X chromosomes and all the other chromosomes, as well, should be present in a homozygous condition, as the material consists of old inbred strains. The observation of a single heterochromatic chromosome per cell in the present material raises many interesting questions.

The fact that this chromosome is present in a single state suggests that it does not belong to the normal chromosomal make-up of this material but that it has originated quite recently through some kind of change. This opinion is, of course, supported by the fact that no heteropycnotic chromosome of this kind was found in normal tissue cells. The change which has given origin to this heteropycnotic chromosome must be a very unusual kind of change. Such chromosomes are not a common and well-known result of ordinary chromosome structural changes and the present material is not characterized by the occurrence of extensive chromosome structural changes in other respects. Under these circumstances it is very remarkable that the same kind of change has taken place in practically all the tumours studied (concerning the interpretation of the single exception, see below). It would be very difficult to understand this situation unless we assumed that this type of cytological change is causally connected to the origin of this type of mammary tumour.

In principle two different types of interrelation might be considered to exist between these heteropycnotic chromosomes and the origin of malignancy in the mammary tumours, *viz.*: *either* the change to malignancy is the cause of the appearance of heteropycnosis in these

chromosomes, *or* the heteropycnosis is an expression of a chromosomal change that has caused the malignancy.

It is true that the malignancy might produce physiological changes in the cells that could be expressed at the chromosome level. When this occurs, however, we should expect that the two homologous chromosomes would become influenced in the same way, as they are of the same constitution and are located in the same physiological environment. For this reason, the occurrence of heteropycnosis in a single member, only, of a chromosome pair suggests that the primary change was in the chromosome itself. Thus, it seems reasonable to suppose that the origin of malignancy in this type of tumour is due to a change of a special kind in a chromosome leading to heteropycnosis of the chromosome in question.

Alternatively, it is possible that the change to heteropycnosis produced a pre-malignant state of the cells from which they, through other changes, could be very easily transformed to a truly malignant state. In any case, the essential point of these results is that they suggest that a change to heteropycnosis in this chromosome has played an essential role in the origin of malignancy; either this chromosomal change was in itself sufficient to produce malignancy, or it caused a pre-malignant state.

As mentioned above some tumour cells in our material were tetraploid and had two heteropycnotic chromosomes. Most probably this tetraploid material has originated from diploid tumour material with one heteropycnotic chromosome per cell through a chromosome doubling by endomitosis or a similar process. Such changes are of common occurrence in tumour material (*e. g.* LEVAN and HAUSCHKA, 1953).

3. The 39-chromosome tumour

Two different interpretations may be presented to explain the exceptional tumour that had only 39 chromosomes and which was lacking the heteropycnotic chromosome:

(1) Originally this tumour might also have been furnished with a chromosome of this heteropycnotic kind in its stem-line, but owing to some mitotic irregularity this chromosome was lost later on. Even if the malignancy was originally conditioned by the presence of the heteropycnotic chromosome, secondary changes that contributed to the malignancy might also have occurred during the tumour progression making it possible for the tumour to remain malignant even after the

loss of the heteropycnotic chromosome. A continued malignancy of the tumour after the loss of the heteropycnotic chromosome would be easy to understand on the basis of the idea (mentioned in the previous paragraph) that the change to heteropycnosis might only have produced a pre-malignant state from which the cells are easily transformed to malignancy through other changes.

(2) Alternatively, it might be suggested that the heteropycnosis of the chromosome in question leads to its physiological inactivation and that for this reason a similar change of the gene balance as that caused by the heteropycnosis might also result from the simple loss of a chromosome of this kind. As this change in the physiological gene balance may result in malignancy, this property may be acquired either through the complete loss of a chromosome of this kind or through its turning heteropycnotic. It is known (see *e. g.* the review by HEITZ, 1956) that heterochromatic chromosomes or chromosome regions often have only a low degree of genic activity. — This second interpretation of the origin of the 39 chromosome tumour does not appear to us, however, to be as probable as the first interpretation, because it does not appear likely that as simple a change as the loss or inactivation of a chromosome would be sufficient to produce malignancy.

4. The origin of heteropycnosis and malignancy

An important question raised by our present observations is that concerning *the nature of the change that causes heteropycnosis* in one member only of the two chromosomes of a pair. In our opinion two different interpretations are worth considering, *viz.*: (1) The heteropycnosis might be the direct visual expression of a virus infection in a chromosome, the whole chromosome being charged with virus or pro-virus[1] particles to such an extent that its staining properties are changed. (2) Alternatively the heteropycnosis might result from a kind of position effect by means of which a localized change in a small chromosome region (gene mutation, small structural change or the attachment of a pro-virus) influences the contraction and staining properties of the whole chromosome.

It is known that certain viruses are able to grow inside the nuclei of mammalian cells. Examples of this are the human so-called adeno-viruses, also known as the RI-APC group (see *e. g.* MORGAN, HOWE, ROSE and MOORE, 1956, and BLOCH, MORGAN, GODMAN, HOWE and

[1] Concerning the distinction between virus and pro-virus see LWOFF (1953).

ROSE, 1957). Another example is the virus causing the infectious warts of the human skin (BLOCH and GODMAN, 1957). The adenoviruses produce Feulgen positive crystals inside the nuclei. Electron microscopic studies show these crystals to consist of close-packed virus particles (MORGAN *et al.*, 1956; BLOCH *et al.*, 1957).

Other results of interest in the present connection are those obtained from recent studies on the interaction between bacteria and certain types of bacteriophages called temperate phages. Thus, we have here the phenomenon called *lysogenization* (LWOFF, 1953; JACOB and WOLLMAN, 1957), which implies that a bacteriophage (or more exactly the genotypical material of a phage) becomes attached to a specific locus of the bacterial chromosome. The attached phage material (which is called pro-phage) is regularly reproduced and transmitted to the daughter chromosomes very much in the same way as if it had been a gene of the bacterial chromosome. Occasionally (in a low frequency of the bacteria) the phage is liberated from the chromosome, it then reproduces in the cytoplasm and causes lysis of the bacterium. The phages liberated in this way are able to infect other sensitive bacteria, to reproduce in them and cause their lysis. Bacteria that carry a prophage attached to their chromosomes are themselves immune to infection by phages of the same kind.

When a sensitive bacterium is infected by a temperate phage, it usually undergoes lysis and liberates a high number of such phages. In a small number of the infected bacteria, however, the phage becomes attached to the bacterial chromosome and is again transformed to a pro-phage. A bacterium in which this has occurred gives rise to a new strain of immune bacteria carrying pro-phages.

It has been demonstrated that such temperate phages are able to bring small pieces of the bacterial chromosomes with them when they move from one bacterium to another. Such chromosome pieces can be implanted in the new bacterium and their genes can through a process similar to crossing over be transferred to the chromosome of the bacterium. This process, which is one of the ways in which gene recombination in bacteria takes place, is known as *transduction* (see *e. g.* the review by HARTMAN, 1957). Transduction has turned out to be a very useful method for the mapping of bacterial chromosomes and for the analysis of genic structure (DEMEREC, 1957).

It has been realized by some authors that similar interactions between viruses and chromosomes as are known from bacteria might occur also in higher organisms and that phenomena of this kind might be of im-

portance for an understanding of certain cancer problems (*e. g.* LWOFF, 1953; JACOB and WOLLMAN, 1957). But, as JACOB and WOLLMAN state (*l. c.*, p. 494): »It must only be realized that no evidence in favor of the existence of proviruses in the cells of animals or plants has so far been obtained.»

Naturally, the results of the present study are not sufficient to prove that the chromosomal heteropycnosis and the transformation of the cells to malignancy in these tumours was caused by the attachment of a cancer virus to a chromosome. It seems to us, however, that a hypothesis of this kind gives us the most reasonable explanation of our observations.

In our hypothesis it is supposed that the virus (the milk factor) is *normally* living somewhere else in the cell, probably in the cytoplasm (but possibly also in the nucleus, without being attached to a chromosome). It has the ability of becoming attached to a chromosome, but this event occurs only very rarely. The virus-infected chromosome is transformed to the heteropycnotic state. As the attachment of the virus to a chromosome is a very rare event, the probability that two chromosomes in the same nucleus will be changed in this way will be very small. For this reason the cancer cells contain a single heteropycnotic chromosome only. By its presence on the chromosome the virus causes a change of the cell physiology leading to malignancy. It is quite possible that the component that is attached to the chromosome, reproduced with it and transferred in this way through several cell generations, may not consist of complete virus particles but rather their genotypic material, the pro-virus.

As mentioned above two alternative interpretations may be suggested to explain the special character of the observed heteropycnotic chromosomes. In both cases the primary change in the chromosome might have been the attachment of the virus to it. According to the first alternative the attached virus has the ability to spread along the infected chromosome, but is unable to spread from one chromosome to another. It multiplies and is distributed along the chromosome, which is charged with virus material, to such an extent that the staining properties of the chromosome is changed.

According to the second alternative the attached virus remains localized in a small region of the chromosome; it may even be a single virus individual that is attached to a specific locus (a behaviour known from bacteriophages that are attached to the bacterial chromosomes). If this is the case, the heteropycnosis must be due to a physiological

influence from the attached virus, or from the locus changed by its presence, that extends over the whole chromosome in question, but which must be unable to extend to other chromosomes in the same nucleus (as the other member of the same chromosome pair is found to remain unchanged). An influence of this type would obviously be a kind of *position effect*, a presence or absence of heteropycnosis in certain chromosome regions being due to a localization in the same chromosome as a changed locus (or chromosome region) or in another chromosome. This represents an influence of a type which, as DARLINGTON (1956) has expressed it, is »canalised along the chromosome».

There exists another cytological phenomenon which appears to support the assumption that position effects influencing the degree of heteropycnosis of a chromosome region may occur. In many species the prophase chromosomes (of both meiosis and ordinary mitosis) show a positive heteropycnosis close to the centromere combined with a gradual decrease of the degree of stainability and thickness of the chromosomes towards their ends. DARLINGTON (1933), who studied this phenomenon in *Agapanthus umbellatus,* expressed the opinion that the difference in appearance of the chromosome regions in this morphological gradient was caused by their position relative to the centromere, which controlled the whole process. The same interpretation was later expressed by LIMA-DE-FARIA (1952, 1953) who studied this phenomenon in rye and *Agapanthus.* In the absence of experimental studies on this question (by means of inversions and other chromosome structural changes) it is difficult to know, however, whether the gradient (a term introduced in this connection by LIMA-DE-FARIA) is due to a physiological influence of the centromere on the contraction and stainability of the other chromosome regions or whether it is due to other circumstances.

In any case we may well consider the interpretation that the heteropycnosis in our mouse tumours may be due to an influence of a small localized change which has a physiological action extending over the whole chromosome. Naturally, the localized change need not necessarily be the attachment of a virus (or pro-virus) particle to the chromosome, but it might also be a specific mutation or a small structural change of a specific kind induced by the virus. The assumption of such highly specific mutations (or similar changes) does not appear probable, however. For this reason we think that an attachment of the virus to the chromosome (such as is known from bacteria) is a more probable hypothesis.

At present it is impossible to evaluate the relative probability of the

two alternative hypotheses presented above — *i. e.*, whether the heteropycnosis in our case is due to a charge of the whole chromosome with virus particles or whether it is due to a physiological action originating from a localized change and extending over the whole chromosome.

Another question of interest in connection with the effect of the milk factor in producing cancer is its tissue specificity, its action being limited to the mammary glands. This may only be a special case of the tissue specificity often exhibited by viruses in general. At the base of such problems lies the question of the nature of the processes of tissue differentiation during embryogenesis. Certain recent studies indicate that nuclear changes may be of some importance for the tissue differentiation. This appears very clearly from the work by KING and BRIGGS (1957) on nuclear transplantations in frog embryos. There are also a number of recent investigations, especially on dipteran salivary glands, showing that various chromosome regions or bands are present in different functional states in different types of cells and during different developmental stages (BEERMANN, 1952 a and b, 1957; MECHELKE, 1953; BREUER and PAVAN, 1955).

Under these circumstances it seems reasonable to suppose that in the mammary tissue of the mouse there are certain chromosome regions (or gene loci) that are in a state of high physiological activity. Probably the activity in one of these loci is of such kind as to make it especially susceptible to attack by the cancer virus. For this reason the cancer virus may in a small percentage of the cells become attached to the chromosome at this locus. After that it will spread from this locus over the whole chromosome in question (if we accept the first one of our two alternative interpretations in this regard).

According to the interpretations given here the milk factor would be non-pathogenic in the ordinary virus state. It is only after its transformation to a pro-virus, attached to a chromosome, that it causes a disease by provoking malignancy (or alternatively pre-malignancy) in the cell carrying it. It is known that bacteria carrying pro-phages may have their properties changed by the presence of these pro-phages on their chromosomes. Thus, it was found that the production of toxin in *Corynebacterium diphteriae* is caused by its lysogenization by a temperate phage (see the review by JACOB and WOLLMAN, 1957, p. 493).

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SUMMARY

A chromosome study has been made of 19 spontaneous mammary carcinomas from 11 dba and 7 C3H mice. These strains are known to contain the milk factor, a virus or virus-like agent of essential importance for the origin of these breast tumours. In 16 of the tumours the chromosome number was 40 with but little variation. One tumour had 39 chromosomes in all of the cells counted. Another tumour had a mixture of cells with 40 and 80 chromosomes in about equal proportions, and a third tumour had 80 chromosomes in almost all of its cells. Thus the chromosomal variation found in these primary tumours is much narrower than the variation usually found in ascites tumours. On the other hand, the mammary tumours showed a chromosomal heterogeneity never found in normal liver and spleen tissue.

A conspicuous heteropycnotic chromosome was found in all the tumours except the one with 39 chromosomes. This chromosome was represented once per cell in the diploid material and twice in the tetraploid.

It is supposed that the chromosomal peculiarities of these mammary tumours are due to their origin through the action of a cancer virus. This virus may have the ability to attach itself to a chromosome and as this is a very rare event only one chromosome per cell will be infected by the virus when such an infection occurs. The virus-infected chromosome becomes heteropycnotic *either* as a simple consequence of an extensive charge of the chromosome with virus (or pro-virus) particles *or* as the result of a physiological action from a localized virus particle of such kind that it works only on the material in the same chromosome (*i. e.*, a position effect). When the virus has become attached to the chromosomal material, it causes a change of the cell physiology leading to malignancy. In normal cells the virus exists elsewhere in the cell, perhaps in the cytoplasm. Reference is made to the fact that similar close interactions between viruses and chromosomes are known from the lysogenization of bacteria by temperate bacteriophages.

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PERMANENT PREPARATIONS FROM ENDOSPERM CELLS FLATTENED IN THE LIVING STATE

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THE endosperm of various plants has turned out to be a very useful material for studies on chromosome behaviour in living cells (BAJER and MOLÈ-BAJER, 1955). The special feature of the endosperm, which makes it more suitable than other plant tissues, is that in many species there is no formation of cellulose walls during the early stages of development of the endosperm. These cell walls that are present in other types of plant cells cause serious disturbances when phase contrast microscopy is used. Furthermore, the absence of such cell walls in the endosperm makes it possible to flatten the cells considerably simply by means of surface tension. When the cells are properly flattened it is possible to study the behaviour in detail of many individual chromosomes in each mitosis from prophase until telophase. Figs. 1—2 shows two mitotic stages from a flattened cell of this kind. The pictures are phase contrast photographs from a living cell.

Many different species can be used in studies of this kind. A list of species tested in this respect was given by BAJER and MOLÈ-BAJER (1954). Very suitable materials for this purpose are *Leucojum aestivum* and *Haemanthus Katherinae*.

When the course of mitosis in living cells is to be studied the material is prepared in the following way. An ovule in a suitable stage is opened and the content of the embryo-sac is pressed out on a cover glass smeared with a thin layer of agar (0.4—0.5 %) containing glucose (3—4.5 %). After removal of the excess of endosperm liquid the surface tension will flatten the cells (BAJER, 1955).

This technique of flattening the cells is quite different from that applied at the preparation of ordinary squashes from fixed material. In the squashes the content of a dead cell is violently spread into an

artificial arrangement, which does not permit much conclusions concerning the position and behaviour of the chromosomes in the living state. In the living endosperm cells, flattened by the action of the surface tension, the mechanical conditions in the cell are changed relatively slowly, in a way which permits a gradual adaptation of the mitotic apparatus to the flattened state. When such cells are properly flattened, it is even possible for most of them to pass through the whole process of mitosis in this state, entering into mitosis from the resting stage and completing the process in a normal way.

It might be of some value to be able to produce fixed and stained preparations also from cells which had been flattened in this way in the living state.

Unfortunately the layer of agar, to which the cells are attached, is liable either to be dissolved or to become detached from the glass during fixation, staining and dehydration. This difficulty, however, can be overcome by mixing the agar medium with gelatin. Gelatin and agar are dissolved separately to give one per cent solutions (containing the desired quantity of glucose, in the case of *Haemanthus Katherinae* 3.5 %). These two solutions are mixed in equal parts just before use. The cells continue mitosis very well on this medium, too. It was not checked, however, in our experiments, whether the cells were able to continue division for as long a time as they do on agar without gelatin.

As gelatin is made insoluble by some of the components used in fixatives, such as formalin and chromic acid, the gelatin will make the gel layer much more stable towards the staining and dehydration procedures. NEWCOMER (1938) found that a culture medium containing gelatin besides the agar was suitable, when slides of growing pollen tubes were to be fixed for the production of stained, permanent preparations.

As fixative we used a modification of NAVASHIN's mixture. We used the modification suggested by MÜNTZING (1933), which we, however, diluted with an equal quantity of distilled water.

We stained our preparations according to the Feulgen procedure. In order to decrease the risk of dissolving or removing the gel layer during the hydrolysis we performed this operation at room temperature by means of an alcoholic solution of hydrochloric acid (3 parts 95 % alcohol mixed with 1 part concentrated HCl, sp. gr. 1.19). A Feulgen hydrolysis by means of alcoholic solutions of HCl has earlier been used by various authors (*e. g.* JOHNSON, 1945).

It is a well-known fact that the optimum time of hydrolysis for the

Feulgen reaction is different depending on the type of fixation used. When chrome-acetic-formalin fixatives are used, the hydrolysis should be longer than in the case of fixatives of the acetic-alcohol type. It is also well-known that the former type of fixatives permits a much wider variation to be used in the time of hydrolysis than the latter type. We found that with the fixation used by us the hydrolysis in alcoholic HCl could be varied from 2 hours until at least 12 hours without much change in the quality of the staining. Four hours of hydrolysis appear to be a suitable time.

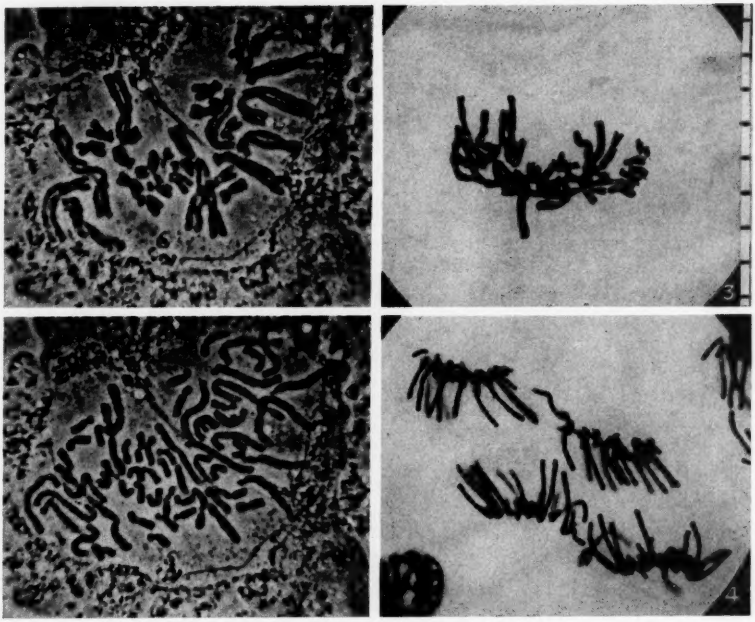
It seems that the Feulgen stain develops more slowly after this alcoholic hydrolysis than it does after an ordinary aqueous hydrolysis. We have not, however, made exact quantitative comparisons of the possible difference. In any case, the staining should be permitted to continue for at least 4 hours. Twelve hours of staining give a good result. (If there is a real difference in the time of stain development from that found at aqueous hydrolysis, this might be due to a masking of some of the aldehyde groups by acetal formation during the alcoholic »hydrolysis». The acetal groups will probably react more slowly with the staining solution.)

After the fixation but before the hydrolysis we applied a treatment of the material with a mixture of equal parts of a decinormal (=0.65 %) solution of potassium cyanide and a 2 % solution of magnesium sulphate. This cyanide treatment is used by ÖSTERGREN (not published before) as a routine method before the Feulgen hydrolysis, when chromic fixation has been used, in order to get a completely unstained cytoplasm. When oxidizing fixatives are used there are produced a few aldehyde groups in the cytoplasm and the cell walls. These may cause a slightly stained background, when the Feulgen method is applied. Such undesirable aldehyde groups can be effectively destroyed by a cyanide treatment of this kind for one to two hours. The material must be carefully rinsed in water after the cyanide treatment before it is hydrolyzed. The mixture of KCN with $MgSO_4$ was recommended by SCHULEK (1925) to be used in analytical chemistry for the quantitative determination of formaldehyde.

Haemanthus Katherinae was used as experimental material for this study.

The following method was found to be suitable for the production of fixed and stained preparations of endosperm cells flattened in the living state.

1. The endosperm cells are spread on a cover glass smeared with a



Figs. 1—4. Mitosis in the endosperm of *Haemanthus Katherinae*. — Figs. 1—2. Phase contrast photographs from a flattened living cell. Two different mitotic stages from the same cell. — Figs. 3—4. Photographs from a permanent Feulgen stained preparation of cells flattened in the living state. — ×530.

thin layer of a mixture containing $^1/_2$ % agar, $^1/_2$ % gelatin and 3.5 % glucose. The excess of endosperm liquid is removed and the surface tension flattens the cells. The preparation is arranged as a moist chamber in order to prevent drying before the fixation (BAJER, 1955).

2. After a while the cover glass with the cells is fixed in the chrome-acetic-formalin fixative of MÜNTZING (1933, p. 131) diluted with an equal volume of distilled water.

3. The cover glass is left in the fixative for 12 hours to some days.

4. It is rinsed in water and treated for one hour with a solution consisting of equal volumes of decinormal potassium cyanide and 2 % magnesium sulphate. (Caution should be used as the solution gives off some fumes of hydrogen cyanide, which is very poisonous.) After this treatment the cyanide is carefully washed out by several changes of water.

5. The cover glass is hydrolyzed for about 4 hours at room temperature in a mixture of 3 parts of 95 % alcohol and 1 part of concentrated hydrochlorid acid.

6. It is dipped in water and stained for several hours (12 hrs gives a good result) in fuchsin sulphurous acid (the ordinary Feulgen reagent).

7. The preparation is washed in SO_2-water, dehydrated through an alcohol series, passed through an alcohol-xylol mixture to xylol and is mounted in the usual manner.

Figs. 3—4 demonstrate the appearance of some cells in preparations produced by this method. For comparison we give Figs. 1—2 which show two different mitotic stages from a living cell photographed by means of a phase contrast microscope.

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ACCELERATED EVOLUTION OF CANCER STEMLINES FOLLOWING ENVIRONMENTAL CHANGES

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I. INTRODUCTION

CHROMOSOME analyses always have revealed heterogeneity of cancer cell populations, providing a high frequency of genotypical changes. In the tumour, genotypic equilibria are constantly formed and again upset; stemline shifts are induced by selective pressure, those genotypes best adapted to the prevailing environment always being favoured. Thus, on the level of individual cells cancerogenesis and cancer progression in several respects parallel the evolution of organisms.

However, to mention one difference only, the absence of the strictly conservative meiotic process from somatic cell lineages will endow these with greater evolutionary flexibility. On the other hand they lack the possibility of recombination at meiosis available to the majority of organisms.

The present paper deals with numerical and structural chromosome changes observed in some clonal tumours consequent to their establishment from a mouse tumour with a long history of serial transfer. It is shown that the change in environment involved in the clone production induces the creation of stemline changes in a tumour which for a long period has maintained the same stemline. The clonal production acts as a stimulant of the evolutionary rate.

After FURTH and KAHN (1937) had shown that mouse leukemias were transferable by intravenous inoculation of one single cell, several cases have been reported of clonal production *in vivo*. Thus, ISHIBASHI (1950) and HOSOKAWA (1950) were able to transfer the Yoshida rat sarcoma by intraperitoneal injection of one cell. The latter writer determined what percentage of single cells present in the pipette will actually reach the peritoneal cavity on inoculation. The chromosomes of Yoshida sar-

coma clones were examined by SATO (1952). The production of clones from other rat tumours have been reported by Japanese workers. MAKINO (1956) produced clones of the HIROSAKI sarcoma and described five different stemline karyotypes, and YOSHIDA (1956, 1957) reports clones from a hepatoma ascites.

HAUSCHKA (1953 a and b) was the first one to establish clones from mouse ascites tumours, *viz.* some 20 clones of the hypotetraploid Krebs 2 and Ehrlich carcinomas, the cytology and functional characterization of which were studied by HAUSCHKA and LEVAN (1958). Additional clones of the hypoteteraploid Ehrlich tumour were made by KAZIWARA (1954), who, however, failed in producing clones of the hyperdiploid Ehrlich. It was at first thought that clones would not by viable in the mouse on that ploidy level, but LETTRÉ and QUERNER (1954) and QUERNER (1955) were successful in producing clones of the hyperdiploid Ehrlich. Chromosome analyses of these clones were given by QUERNER (1955).

From a solid line of the mammary carcinoma S3A E. KLEIN (1955) established new solid tumours by subcutaneous inoculation of one cell.

II. MATERIAL AND METHODS

1. The mice used

The mouse strains used as host animals for the tumour cells were C3H and DBA and white hybrid mice of unknown origin. The age of the young at the time of inoculation varied between two and ten days. Animals two to three days of age are preferable not only because of their weak reaction against the inoculated cells but also because the ease with which the cell inoculations are performed.

2. The tumour used

The tumour with which the present investigation was carried out originates from the laboratory of dr. C. LANDSCHÜTZ in München in 1952. I have had the opportunity to examine the original slides made by dr. LANDSCHÜTZ and I am able to state that no visible change in the stemline chromosome complement has taken place from 1952 to 1958. It is evident from the chromosome analyses made by TJIO and LEVAN (1954) that the tumour is a substrain of the old Ehrlich carcinoma, which has been carried in laboratories for many years.

We obtained the tumour in 1953 by the courtesy of dr. KLEIN, Stockholm, who had just received it from dr. LANDSCHÜTZ. It has been carried

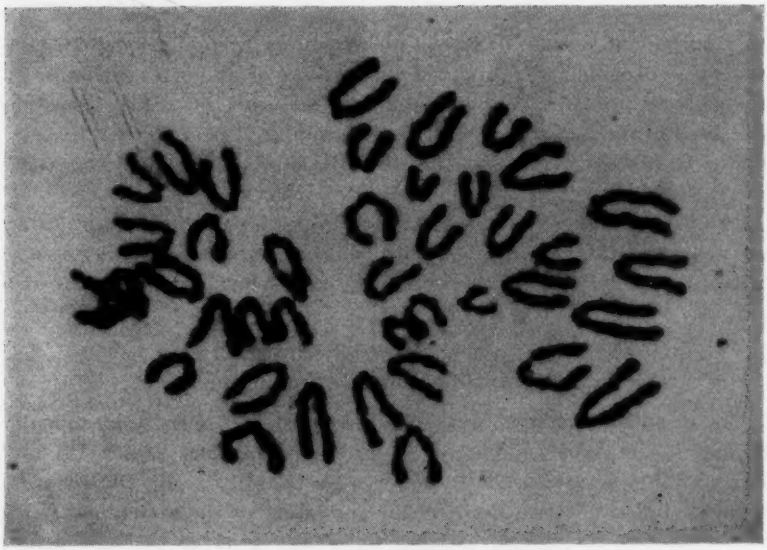

Fig. 1. Photomicrograph of a normal mouse cell in metaphase, $n=40$. — ×2400.

since then as ascites in hybrid white mice, C3H and DBA with equal ease.

3. The isolation and inoculation of single cells

Ascites fluid containing cells of the Landschütz tumour, grown for 7—9 days in its host, was diluted with supernatant of the same tumour. The latter was collected from other animals and was made cell free by triple centrifugation. With this method uncontrolled effects of unknown substances on the cells were avoided. The dilution was made in three steps. At first $^1/_8$ droplet ascites fluid was added to 10 ml medium. At the second step one droplet of this suspension was added to 10 ml medium. At the third step the dilution was adjusted so that most microdroplets of the resulting suspension held either one cell or none. The original undiluted ascites fluid was kept all the time in ice water and no working suspension was used for more than three hours.

By aid of an Agla micrometer syringe eight microdroplets of the third dilution were placed in a row on the slide, which had been carefully cleaned to make the droplet as flat as possible.

When a droplet had been shown by microscopic inspection to contain only one undamaged cell, it was picked up by a capillary pipette, the upper end of which was provided with a piece of an open latex tube. As no micromanipulator was available at that time the entire operation was made by hand which with some training was made quite easily. It was controlled microscopically that the pipette each time contained one cell.

The point of the pipette was inserted into the peritoneal cavity of an infant mouse, the cell with the fluid being injected by a slight pressure of the latex tube. The latter procedure was preferably handled by two persons.

4. The examination of chromosomes

The established ascites tumours were transferred serially by inoculation of 0.2 ml ascites, diluted $^1/_{10}$ with physiological saline. On day seven after inoculation the tumours were ususally ready for chromosome study. In order to improve the spreading of the metaphase chromosomes a colchicine pretreatment was routinely given 18 hours before fixation. A colchicine solution containing 5—10 γ colchicine was injected intraperitoneally per 10 g body weight, which would give a colchicine concentration of $1250—2500 \cdot 10^{-9}$ mol/l in the animal. At the fixation a sample of the ascites fluid drawn with a small glass pipette was blown directly into a tube with about five times its volume of acetic orcein (2 % orcein in 60 % acetic acid). The tube was shaken for obtaining an instantaneous fixation of the cells. After 2—3 minutes small droplets of this mixture were placed on slides and squashed.

At the preparation of the idiograms each chromosome has been drawn in the center of the viewfield with the aid of a camera lucida, the resulting magnification being $\times 3000$. The chromosome counts upon which the histograms are based are made from completely analyzable plates and the counts are exact.

III. RESULTS

At the first series of single cell isolations white hybrid mice of unknown origin were used as recipients. This series included 86 infant animals out of 18 litters. Thirty days after the inoculation samples of the peritoneal fluid were examined. In two of these cases dividing cells were found. It could be unequivocally established by analysis of the chromosome complements that these cells belonged to the Landschütz

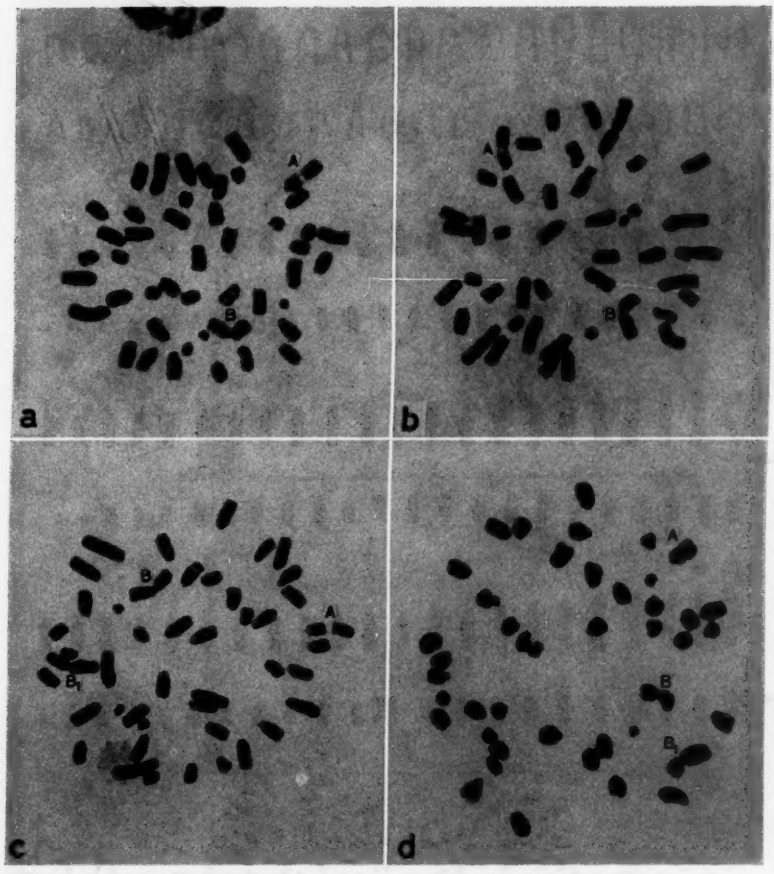

Fig. 2. Photomicrographs of metaphase plates from:

a: Landschütz sarcoma I, $s=46$,
b: clone 21/119, $s=47$,
c, *d*: clone 30/172, $s=45$; *d* has 44 chromosomes. $\times 1070$.

tumour and not to the host animals. As first demonstrated by BAYREUTHER (1952), the hyperdiploid Ehrlich, of which the Landschütz tumour is a strain, has the following distinctive chromosomes, not present in the mouse idiogram: one large telocentric chromosome with a secondary constriction (the A chromosome), one large metacentric

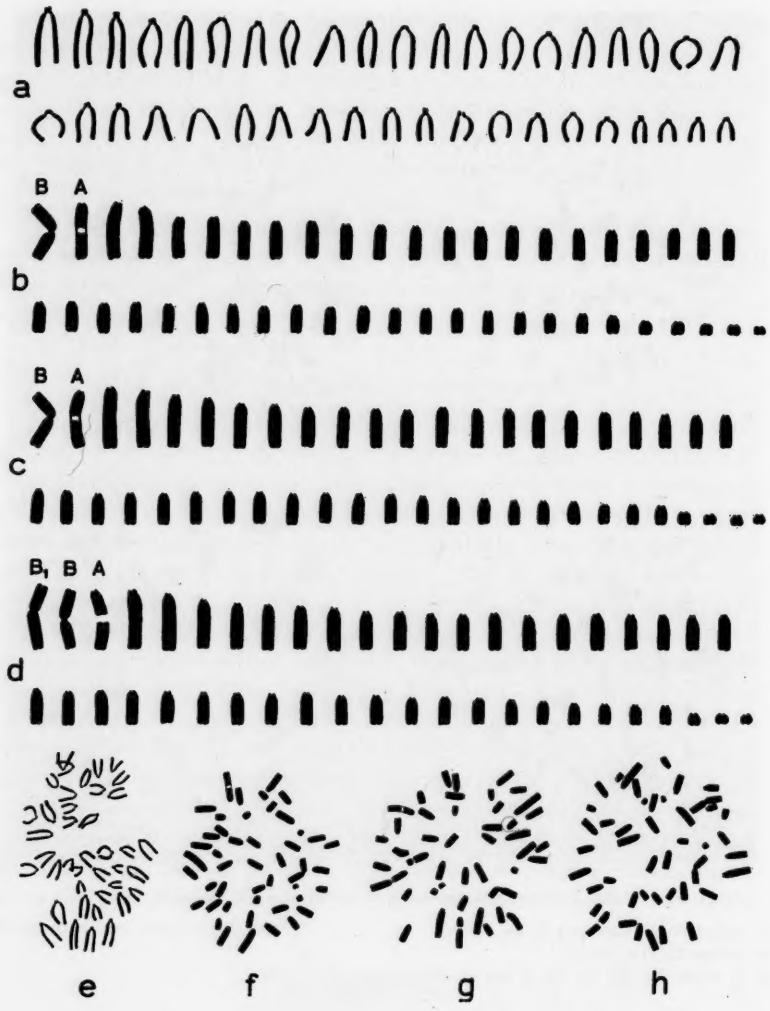

Fig. 3. Idiograms of:

a, e: normal male mouse cell, $n=40$;
b, f: a cell from the Landschütz sarcoma I, $s=46$, one metacentric chromosome;
c, g: a cell from the clone 21/119, $s=47$, one metacentric chromosome;
d, h: a cell from the clone 30/172, $s=45$, one metacentric and one submedially inserted chromosome.

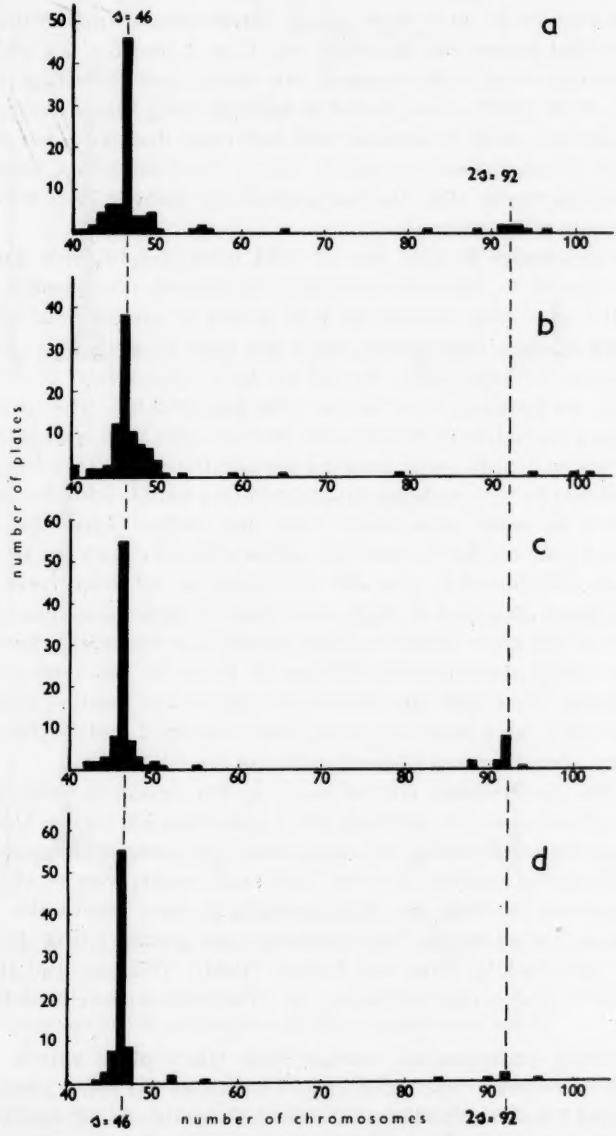

Fig. 4. Histograms showing the chromosome number distribution of four clones with $s=46$. The chromosomes of 100 plates were counted in each case. All counts are exact.

chromosome (the B), and three minute chromosomes, smaller than any of the normal mouse chromosomes (*cf.* Figs. 1 and 3 *a*, 2 *a* and 3 *b*).

The animals were again sampled two weeks later. This time 19 animals out of 86 (22 %) were found to have dividing tumour cells. None of these animals with the tumour cells had more than some hundredths of a ml of fluid in their peritoneal cavity. New samplings were tried eight and ten weeks after the inoculation. No tumour cells were now found in any of the animals.

In another series 35 C3H and 37 DBA mice, one to three days old, were inoculated. In this series altogether six animals developed tumours. Four DBA mice were coming up with plenty of ascites fluid after 26, 28, 35 and 40 days, respectively, while two more DBA animals exhibited solid tumour formation after 45 and 49 days, respectively.

As soon as possible each tumour was transplanted. The ascites tumours were immediately transferable, but the solid ones were minced in saline solution before being injected intraperitoneally. This resulted in solid tumours in the omentum and in addition ascites with free tumour cells. New passages were made from the ascites. After the second passage all tumours have grown as ascites and since then the six clones have been maintained *in vivo* and also stored in the deep-freeze.

My original plan was to carry two lines of each clone, one in DBA and one in C3H mice. These two lines would be periodically checked for possibly arising chromosomal differences. However, this turned out not to be feasible. New C3H lines repeatedly were made, starting from DBA lines, but they have been lost every time. Finally, I had to give up the C3H lines altogether and to concentrate on the DBA lines.

The first chromosome control made in the second to third passage showed all the clones to maintain the Landschütz karyotype. One clone had many hypertetraploid cells but most cells were still hyperdiploid. A second careful analysis in which only exact counts were recorded was made between the 24th and 28th passage. At these counts the cells of four clones had a distribution of chromosome numbers (Fig. 4) similar to that described by TJIO and LEVAN (1954). This material included the clone having a high frequency of hypertetraploid cells at the first control.

Interesting chromosomal changes had taken place within the remaining two clones. The clone 21/119 which at the third passage had the typical Landschütz idiogram with the stemline at 46, had changed its stemline chromosome number to 47 when studied at the 25th passage (Fig. 5 *a*). Thus, one chromosome had been added to the complement.

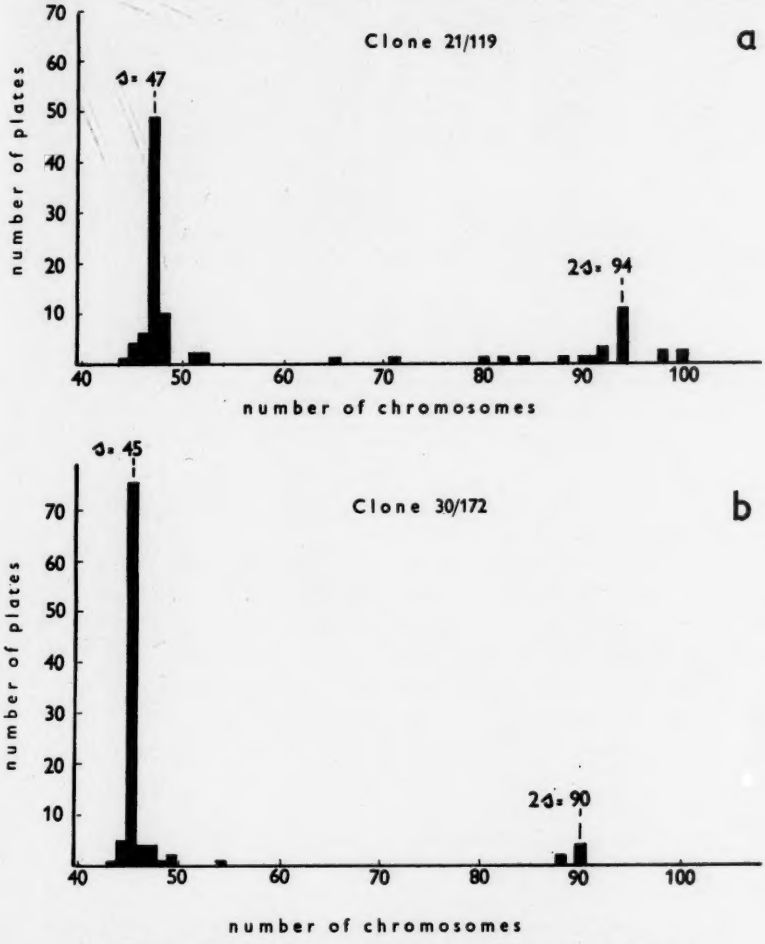

Fig. 5. Histograms showing the chromosome number distribution of:

***a*: clone 21/119, $s=47$;**
***b*: clone 30/172, $s=45$.**
The chromosomes of 100 plates were counted. All counts are exact.

It was not possible to identify this particular chromosome which must be a telocentric one, probably of medium size (Fig. 2 *b*, 3 *c* and *g*).

The second deviating clone, 30/172, with a 46 chromosome stemline at the third passage, had the stemline number 45 at the 24th passage

(Fig. 5 *b*). In this case one easily recognizable metacentric chromosome had been added to the complement. The new chromosome differs from the original metacentric Landschütz chromosome, the B, by having its centromere not quite medially located which makes the identification of the two kinds of metacentrics fairly easy (Fig. 2 *c* and *d*, 3 *d* and *h*). The new metacentric chromosome will be called B_1 in this paper.

The B_1 chromosome evidently corresponds to two telocentric chromosomes, fused at their centromeres. Similar cases of »new» V-shaped chromosomes are known in mouse and rat tumours *cf. e. g.* MAKINO, 1952).

Although in all tumours there is a slight non-disjunction variation in chromosome number around the modal number, the main characteristics of the Landschütz idiogram are maintained, the only deviation being the gain or loss of one or more chromosomes (Figs. 4 *a*—*d*, Figs. 5 *a* and *b*).

Competition experiments

With the view of making a comparison of the competitive capacity between the two deviating clones and the mother tumour a few experiments with cell mixtures were arranged. The following tumours were used in these experiments:

I. Landschütz 1, the mother tumour: s=46, one metacentric chromosome, B, according to BAYREUTHER.
II. Clone 21/119 s=47, one metacentric chromosome, B.
III. Clone 30/172 s=45, one metacentric, B, and one submedially inserted chromosome, B_1.

The following mixtures of equivalent volumes of each of the tumours used were made:

I+II the histogram of the chromosome counts represented in Fig. 6 *a*.
I+III the histogram of the chromosome counts represented in Fig. 6 *b*.
II+III the histogram of the chromosome counts represented in Fig. 6 *c*.
I+II+III the histogram of the chromosome counts represented in Fig. 6 *d*.

The mixtures were injected into adult DBA mice, 0.2 ml in each.

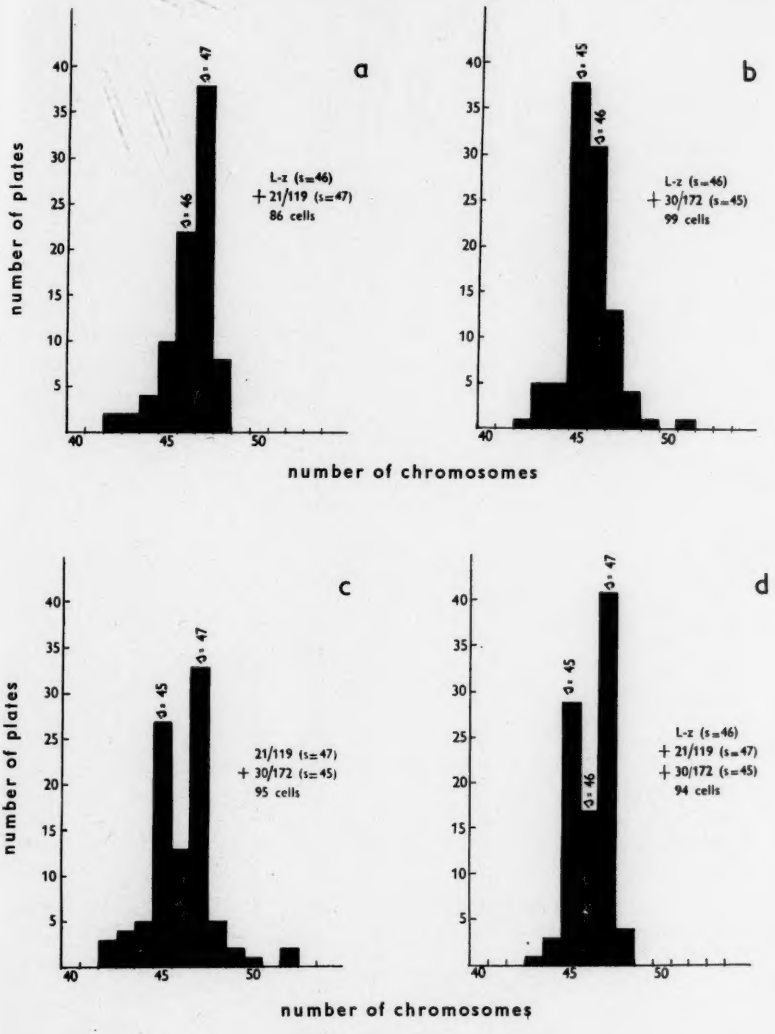

Fig. 6. Histograms showing the chromosome number distribution in the cell mixtures of the original Landschütz sarcoma I and the clones 21/119 and 30/172. Only the diploid parts of the histograms are represented.

TABLE 1. *The distribution of chromosome numbers in tumour cells from mice injected with mixtures of the Landschütz Sarcoma I and two deviating clones.*

Clone	Number of chromosomes: 42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
L-z+21/119	2	2	4	10	27	38	8						2											
L-z+30/172	1	5	5	38	31	13	4	1		1														
21/119+30/172	3	4	5	27	13	33	5	2	1		2													
L-z+21/119+30/172		1	3	29	17	41	4																	

Clone	Number of chromosomes: 66	67	68	69	70 . . .	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	n
L-z+21/119						1							1		1		4							100
L-z+30/172																			1					100
21/119+30/172			1		1								1		1		1							100
L-z+21/119+30/172									1				1				2						1	100

Chromosome counts of the mixed tumours were made on day eight to nine after inoculation. The counts are recorded in Table 1.

The histogram of Fig. 6 *a* has one peak at 47 chromosomes and another somewhat lower peak at 46 chromosomes. Fig. 6 *b* shows a higher peak at 45 chromosomes and another slightly lower at 46 chromosomes. The histogram Fig. 6 *c* representing the mixture of the two deviating clones has two peaks at their stemlines 47 and 45 respectively. Owing to the slight chromosome number variation around the two stemlines the chromosome number 46 is also often found. The analysis of the mixture of the two deviating clones with the Landschütz tumour (Fig. 6 *d*) shows a similar picture, the chromosome number 46 being represented more abundantly in this case.

The frequency of chromosome numbers recorded in the four histograms Figs. 6 *a*—*d* strongly suggests that both of the clones tested have an improved competitive capacity as compared with the original tumour. This probably means that the mitotic rate of the clones is increased. A change of the duration of the mitotic stages in relation to the interphase would give the same result. However, this latter interpretation seems very unlikely.

IV. DISCUSSION

In the first series of the present single cell inoculations hybrid white mice were used as receptors. It is a remarkable fact that although no transplantable ascites tumours developed in this series, no less than 22 % of the animals were found to contain dividing cells of the Landschütz type six weeks after the inoculation. Evidently, the implantation of one single cell resulted in tumour growth in many of the animals, but in every single case immunity reactions of the host animals caused the tumours to regress before they could be transplanted serially.

In the second series, in which the inbred lines C3H and DBA were used as hosts progressively growing clone tumours were obtained in 8 % of the 72 animals inoculated. However, all takes were among the 37 DBA mice, which means a take % of 16 in DBA mice. Contrary to this, the 35 C3H mice inoculated were negative. As mentioned above — even when large inoculates were used — the clones which started in the DBA mice could be grown in C3H only with difficulty, and serial transplants of the clones in C3H mice were difficult to perform.

Even though no definite conclusions can be drawn from the present restricted material there is an indication that the C3H strain has immunity genes affecting the initial growth of single Landschütz tumour

cells. These results are interesting, since the Landschütz strain is generally considered as completely unspecific towards host genotypes. Our experience is that it grows equally well in white hybrid mice, DBA, C3H and the F_1 C3H×DBA.

Within normal tissues *in situ* the mitotic mechanism is stable, all cells having the same genotype and the same chromosome set (cases of polyploidization not mentioned). All tumours are characterized by mitotic disturbances which result in a genotypic heterogeneity, as evidenced by the presence of different karyotypes. However, as long as the environment does not change, old established tumours maintain the same stemline genotype, which has been tried out for that environment and consequently is at a competitive advantage in comparison with the deviating genotypes. If, however, the environment changes, it may well be that a genotype which so far has had a restricted life will be favoured and even take the lead from the old stemline.

Several shifts in tumour stemlines correlated to shifts in environment are known. Thus, the neartetraploid Ehrlich carcinoma, which in Sweden is known to have a stemline with 80 chromosomes, changed its stemline to 76 when brought from Stockholm to Philadelphia (LEVAN, 1956 a). In U. S. A. the lower chromosome number seems to be maintained. The same 80-chromosome tumour has gone through repeatedly a similar shift in number, after a period of growth in hamster (ISING, 1955). Of course, the seemingly identical shift, need not comprise loss of the same four chromosomes. It is of specific interest that the same shift occurred on different occasions in ISING's experiments, which suggests that the Ehrlich tumour carries permanently a ballast of different cell lines beside the stemline. Given the same environmental change, therefore, the same cell line over and over again may take the lead.

A similar change was indicated in a human ovarian cystocarcinoma (HANSEN-MELANDER, KULLANDER and MELANDER, 1956), in which a gradual shift of the stemline number from 58 to 63 was observed.

So far little is known whether genotypic changes are involved in the original change of a cell from normal to malignant. It may be pointed out, however, that the cells of a normal tissue are adapted to ensure a perfect functioning of the tissue. Consequently any mitotic irregularities would be ruled out as disadvantageous.

Cells that happen to escape the control of the host or otherwise obtain an altered environment may no longer function ideally with their normal genotype. Therefore the basis is formed from which selective processes may create cells with new properties, adapted to the new environ-

ment. It is suggestive that the adaptation of normal cells to life *in vitro*, which certainly involves a drastic change in environment is regularly connected with both the acquisition of malignant properties (SANFORD, LIKELY and EARLE, 1954) and a cancer pattern of chromosome behaviour (LEVAN, 1956 b; HSU and MOORHEAD, 1957).

The isolation of a single cancer cell from its surroundings in the tumour followed by the inoculation of this cell into the peritoneal cavity of a new host certainly involves a very pronounced change in environment. In order to survive and start division the cell has to overcome the immunity reactions of the host. It may also be that a factor necessary for development of the cell is missing in one strain of mice but exists in another.

The difficulties of a single cell transplanted *in vivo* may in some respects be compared with those of single cells grown *in vitro*. It is known that the cell in one way or another has to »condition» its medium before mitosis can be entered. SANFORD, EARLE and LIKELY, 1948 were able to obtain mitosis and start a clone from one cell grown in a capillary with only a slight quantity of medium, permitting metabolic products of the cell to accumulate. The same rôle of conditioning the medium is played by a layer of irradiated cells, the feeder layer, in the technique of PUCK, MARCUS and CIECURA (1956).

In the present experiments two periods may be distinguished subsequent to the inoculation of a single cell. (1) *The period of more or less solitary growth.* This period may be quite extended, as shown in the cases in which cancer cells were found $1^1/_2$ month after inoculation, and still no tumour developed. During this period the original cancer stemline may not be especially favourable. Consequently genotypic adjustment may have a chance to become of selective value. (2) *The period of readaptation to growth in ascites form.* After the development of an ascites the stemline, which has become adapted to solitary growth, may no longer be ideal, and a new adaptation to the life as an ascites will start. Of course, there is not necessarily any sharp limits between (1) and (2).

Summing up the above discussion: When cells outside the control of the entire organism are brought into a markedly different environment during a sufficiently long time, the stemline will undergo a process of adaptation. This process may involve changes in several characteristics of the tumours. Among them are the chromosome complement of the stemline and the mitotic activity, factors which are dealt with in the present study.

SUMMARY

The present investigation deals with the production of clones from the hyperdiploid Landschütz tumour of the mouse, which is a substrain of the old hyperdiploid Ehrlich carcinoma.

Six clones, originated in DBA mice, were studied. In two cases the modal chromosome number as well as the rate of the mitotic activity was shown to have changed during the clonal growth. A centric fusion had occurred in the chromosome complement of one of the clones.

The rate of stemline evolution in connection with environmental changes is discussed.

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OBSERVATIONS ON THE SEX CHROMATIN IN THE RAT

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I. INTRODUCTION

AFTER the so-called sex chromatin had been discovered in nerve cells of the cat (BARR and BERTRAM, 1949), the possibility of sex determination by inspection of interphase nuclei attracted great attention. Investigations in various mammals, including man, indicated different »sexability» in different species: (1) sex chromatin evident in all tissues tested, (2) sex chromatin identifyable only in nerve cells, and (3) no sex chromatin identified in any of the tissues examined. To group (1) belong, for instance, the cat (GRAHAM and BARR, 1952), man (MOORE and BARR, 1954) and the Rhesus monkey (PRINCE *et al.*, 1955). Group (2) includes several animals, as the dog and the mink, and group (3), finally, includes both the mouse and the rat (MOORE and BARR, 1953). Because of the popularity as laboratory animals of the latter two species the failure to locate the sex chromatin in them is especially provoking.

My earlier experience with the sex chromatin in man (REITALU, 1957 a) indicated liver tissue as especially favourable for such observations. Consequently, my efforts to identify the sex chromatin in the rat were directed mainly to liver cells. As will be briefly reported below, these investigations resulted in the establishment of safe and clear-cut differences in chromatic structure between male and female interphase nuclei of the rat.

The liver tissue for this investigation was collected from four pairs of ordinary white laboratory rats, each pair comprising one male and one female. The four pairs were, respectively, 5, 25, 50, and 180 days old, the three youngest pairs taken out of the same litter.

Fixation of the tissue and processing of the slides were made simultaneously and uniformly for the two members of each pair, and the samples for fixation were always excised from the same region of the

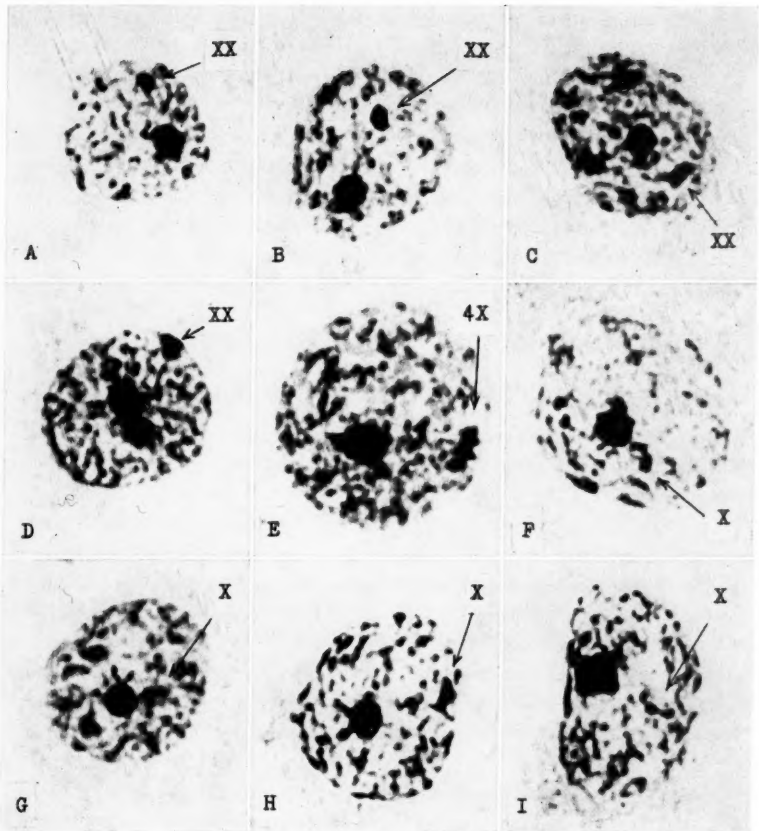

Plate 1. Sex chromatin in liver cells of the rat. A—D, female diploid interphase nuclei; E, female tetraploid interphase nuclei; F—I, male diploid interphase nuclei. — ×1650.

liver. The technique, earlier described (REITALU, 1957 b), was fixation in KAHLE's modified fixative, followed by squashing and differential staining of chromatin and nucleoli in azur A and acid fuchsin. Slides produced in this manner yielded information mainly about shape and position of the sex chromatin, as well as its relations to the nucleolus and to other heterochromatic structures of the nucleus.

Another piece of the same tissue sample was left in the fixative for two hours, transferred to 82 % butanol, and embedded for sectioning (SMITH, 1943). This material was stained in the same way as the squashes. For each pair of animals, sections from both male and female were placed side by side on the same slide, for securing uniform treatment, as far as possible, during hydrolysis and staining. Such slides were used for comparisons of color intensity of the chromatin between the sexes.

II. OBSERVATIONS

The general appearance of the interphase nuclei is different in males and females, the former having relatively lighter nuclei with the stained material more dispersed. All chromatic bodies are thinner, more diffuse, less defined in male nuclei.

There is one characteristic aggregate of chromatin in both sexes, considerably larger than the other chromatic bodies present. It has a striking shape, triangular or irregular, and runs out radially into three or four points. This body consists of the fused heterochromatic parts of two or more autosomes. It is referred to in the present paper as *aa*. It has a deeply saturated colour in female nuclei (Fig. 1 *a*—*c*), and is consistently more faintly coloured in the male, in which its structure is more thread-like (Fig. 1 *h*, *i*).

In the female nuclei another striking body is found, consisting of two parallel rods and somewhat smaller than *aa*. This is a structure, quite regularly present in female but never in male diploid nuclei, and it is therefore considered to represent the heterochromatic parts of the two paired *x* chromosomes. It is designated *xx* (Plate I, A—D). It happens that *aa* and *xx* fuse, forming a large intensely stained body, in which the individual chromosome parts cannot be distinguished (Fig. 1 *d*).

As in man, there is an evident connection between *xx* and the nucleolus. Two chromosome threads start out from the one tapering end of the *xx*, run through the nuclear sap, and attach themselves at one of the nucleoli (Fig. 1 *b*; Fig. 2 *d*, *e*, *f*; Plate I, B). Often, however, the

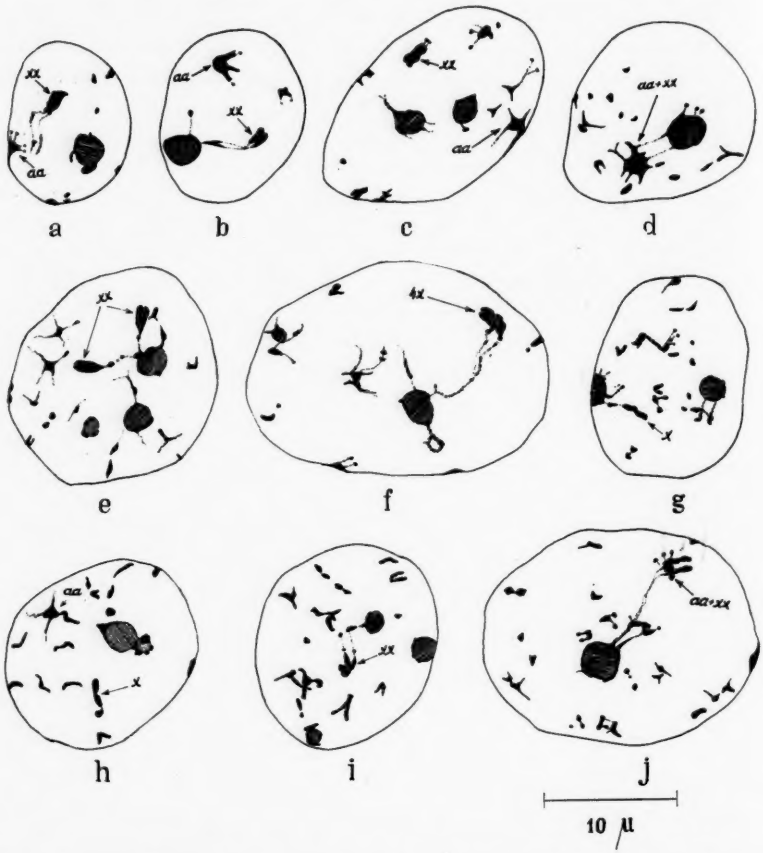

Fig. 1. Some configurations of sex chromatin in liver cells of the rat. — a—d: sex chromatin of female diploid interphase nuclei; e—f: sex chromatin of female tetraploid interphase nuclei; g—h: sex chromatin of male diploid interphase nuclei; i—j: sex chromatin of male tetraploid interphase nuclei. × 2000.

threads are broken, and the *xx* appears free in the interior of the nucleus. In such cases the end of the *xx* structure tapers off into two antenna-like threads, each ending with a small spherical chromatic granule, suggestive of a nucleolus-organizer (Fig. 1 *c*; Fig. 2 *g*, *h*).

The staining quality of the *xx* chromatin is highly variable. While it usually is more deeply coloured than both the *aa* and the other chromatic bodies present, its colour is sometimes quite faint. In the latter

case, the size of the *xx* is enlarged, its structure is more diffuse, and its outlines vague. The antenna-like processes are usually far extended. At the same time the relation of the *xx* to the nucleolus is interesting: the nucleolus is present either in between the two *x* chromosomes (Fig. 2 *a*, *b*) or in their close vicinity (Fig. 2 *c*). It should be noted that such formations were found especially in young animals (five days old).

In male nuclei there is sometimes present beside the *aa* complex one rather extended, somewhat bent, rod-shaped chromatic structure. It may be free or attached to one of the nucleoli by a thread. Since this single rod both in position and general type is reminiscent of one of the two elements of the *xx*, it is safe to conclude that it represents the *x* chromosome (Fig. 1 *g*, *h*; Fig. 2 *i*, *j*; Plate I, F—J). It is evident from the pictures that this single *x* chromosome is somewhat differing in shape from the *x* chromosome as it appears in the paired condition of the *xx* structure of the female. It is longer, less concentrated, narrower, and has two constrictions. The chromatic granule on the antenna is larger both when the thread is free (compare Fig. 2 *g* and *j*), and when it is attached to the nucleolus (compare Fig. 2 *e* and *i*). It is close at hand to think that the heterochromatic part of the *y* chromosome is included in this body, adding to its size.

Both female and male liver tissue frequently contains large tetraploid cells. In the female two identical *xx* bodies are found in the interphase nuclei of such cells. They may exist well-separated from each other (Fig. 1 *e*), or side-by-side (Fig. 1 *f*; Plate I, E), the latter case being exceptional, however. In the male tetraploid nuclei the two *x* chromosomes are often paired into a structure similar to the *xx* of female diploid nuclei (Fig. 1 *i*). But the contact between the two *x* chromosomes is less intimate, an interspace being visible between them. Fig. 1 *j* is a tetraploid male nucleus, in which the *x* chromosomes are in contact with the *aa* complex, forming a mass similar to corresponding formations in diploid female nuclei.

III. DISCUSSION

The resting nuclei of rat liver cells contain a great number of chromatic bodies, ranging from many small bodies to the big *aa* complex, and the *xx* or *xy*, respectively. The number of small chromatic bodies is of the size order of the haploid number in the rat, rather than the diploid number. Normally 16 to 18 such bodies may be counted, and exceptionally more than 20. This number, as well as the shape of many

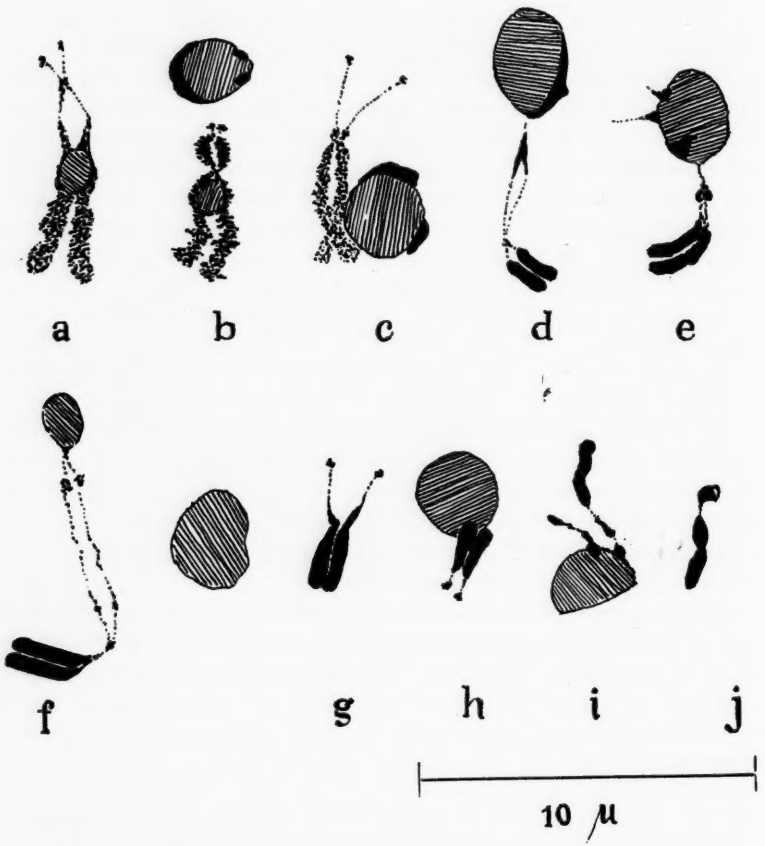

Fig. 2. Some variations in the appearance of the sex chromatin and the relation of the sex chromatin to the nucleoli. — a—h: female diploid nuclei; i—j: male diploid nuclei. ×5100.

of the bodies, suggests that each of them represents two autosomes. It may be that somatic pairing is the rule, although fusions of apparently different bodies also take place.

The large *aa* aggregate is evident in most nuclei. It includes a fusion of two, four, or possibly more chromosomes with large heterochromatic segments. No connection was ever observed between *aa* and any nucleolus.

The *xx* body, regularly found in female nuclei, differs somewhat from the »planoconvex» shape, described for this structure in the cat and in man (BARR and BERTRAM, 1949; MOORE and BARR, 1954). This shape is caused by the close contact of the *xx* to the nuclear membrane. In the rat, the *xx* is usually located in the interior of the nucleus, and when it is close to the membrane, as sometimes happens, it keeps its shape of two parallel rods.

It is interesting that the single *x* chromosome of the male has a modified appearance as compared with conditions in female cells. The hypothetic presence of the *y* chromosome in attachment to the *x* was suggested by REITALU (1957 a) also for the corresponding structure in man. However, at the present stage this suggestion remains a guess.

There is a considerable variation in stainability of *xx* between different nuclei of the same fixation. Thus, the big diffuse *xx* bodies of Fig. 2 *a*—*c*, which are still in close contact with a nucleolus, are probably nearer to the preceding telophase than the rest of the *xx* structures of the same figure, which exhibit deep colour and well-defined outlines, and which are either free from the nucleolus or attached to it by thin, extended threads.

A certain relation exists between the development of the heterochromatic structures and the age of the animals. Because of the higher mitotic frequency of young animals with shorter interphases, the overall background staining makes the heterochromatic bodies less apparent than in older animals, in which the deep resting stage with complete despiralization of the euchromatin makes the heterochromatin stand out. A similar observation concerning the heteropycnotic B chromosomes of *Anthoxanthum* was mentioned by ÖSTERGREN (1947).

A more confusing correlation of the colour intensity with sex was noted, the stainability being weaker in males than in females of the same age. I have found no earlier references to the peculiar difference in nuclear stainability between the sexes, nor are there any observations of sex differences in chemical composition of the chromatin. Differences in heteropycnosis of individual chromosomes are known, however.

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SUMMARY

Morphologic differences in nuclear structure are described between liver cells of male and female rat. The identification of the sex chro-

matin is somewhat hampered by the presence of large autosomal chromatic bodies. As in man, the sex chromatin in the female rat consists of two symmetric parts, often attached to the nucleolus, and interpreted as the heterochromatic segments of the two x-chromosomes. One such structure is present in the male nuclei. It was found that modifications of the stainability of the sex chromatin and other chromatic bodies of the interphase nuclei occurred, correlated with age and sex of the animals.

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CHANGES IN CONSTRUCTION AND POSITION OF CHROMOSOMES DURING THE DIFFERENT PHASES OF MITOSIS AND MEIOSIS

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DURING the last few years the author has carried out a morphological analysis of meiosis and mitosis in different plants. The principal material, however, represents the genera *Tradescantia*, *Lilium* and *Rosa*. The intention of the investigation has been firstly to reconstruct the morphological transformations as thoroughly as possible. The ultimate aim has been to obtain by means of these reconstructions a basis for assessing the underlying mechanics.

Many of the reports in the relevant literature have been confirmed by this study. Some have been corrected or modified. The number of really new facts is a very modest one, if indeed such exist. A comparison between the old and the new reports permit, in my view, the carrying out to a certain extent of a synthesis. Often, however, the combining of available links into a continuous chain demands the introduction of hypotheses. Moreover, a fairly extensive use of hypotheses is inevitable if we endeavour to assess what happens during meiosis and mitosis solely by the aid of photo-microscopic observations. Many of the structures, the closer study of which would seem to be desirable, are of such a degree of size as to be scarcely or indeed not at all accessible.

More detailed descriptions and argumentations concerning the author's views on mitosis and meiosis will be published later in a series of essays. Soon the author hopes to present a work, on the background of chiasma formation and the crossing over phenomenon. This should be followed by an account of the position of the chromosomes during the various nuclear phases and of their so-called wanderings immediately before and after the metaphase stage. A work on chromosomal spiralisation is also planned. As of course a certain time must elapse before the series

planned is ready for publication — there also remain a number of complementary or verificative studies to be made — the author's views are preliminarily summed up in the following points:

1) The mature chromosome consists of a pair of parallel-running units, more or less intimately united with each other; of so-called chromatids. Each chromatid consists in its turn of a pair of parallel-running units; of so-called half-chromatids. Each half-chromatid consists perhaps — at least during certain developmental stages — of two parallel-running units of a lower order, and so forth. It should be impossible to decide how far »back» the system of mutually superior and inferior unit pairs proceeds. Perhaps there is a gradient here; the demarcation and association between the partners in the different pairs is perhaps less and less distinct and more and more intimate, respectively, the further we penetrate into the system. The most subordinate pair perhaps consists of two entirely simple threads (monides) or of two bundles of monides in which the sorting into pairs has not yet begun. A few modern investigations, however, point to a relatively low number of mutually superior and inferior unit pairs.

2) The chromatid, each of the different pairs mentioned under 1) as well as each of their members are to be taken as a chromosome not yet mature. The monide and the undifferentiated monide bundle, respectively, should constitute the initial phase; the half-chromatid, the chromatid and the chromosome should successively represent final stages following on each other. The initial stage's maturity to fully fledged chromosome normally requires several mitoses.

3) The chromosome is more or less strongly twisted around its own axis. This at the same time implies that the two chromatids of the »chromosome spiral» are twined around each other. Each chromatid is — at least during certain developmental phases — in its turn twisted around its own axis. Consequently, the half-chromatids of the »chromatid spiral» are twined around each other during these phases. It is not possible to decide how far the system with the mutually superior and inferior spirals continues.

4) The different units, mentioned under points 1) and 3) as well as the different unit pairs should, during their development from the youngest primordium to the mature chromosome, undergo a spiralisation-despiralisation cycle that calls for several mitoses. If this be correct, each of the spiralisation processes should go towards a maximum. Its conquest would then be followed — possibly not until after a rest — by despiralisation.

5) In addition to the only one-dimensionally twisted systems described above, spiral systems of another character are present during certain phases. It is then that the spiral axis of the unit and unit pair, respectively, has itself the form of a spiral line. The successive units in the spiral complex are 2-dimensionally turned in relation to each other.

6) The twisting conditions can depend on the changes in shape that the thread-building particles or the particle complexes undergo during their reproduction. This is borne out by simple thread-models. Even if this explanation be incorrect, spiralisation can be due to the elements included in the unit pairs possessing sides of different relative ages. The mature and the younger sides should have different length-, contraction- or stretching qualities. Simple twining — one-dimensional twists — may therefore be conditioned by the difference between the central axis and flanks of the pairs of units still attached to each other; systems with a spirally running central axis — systems with 2-dimensional twists — by the difference between the outer and inner sides of the units, now rendered free from each other; and despiralisation by the maturing of the younger sides successively giving them the same characters as those of the older ones.

7) The spiral systems of different order, mentioned under point 2), exert a mutually »deforming influence» on each other. Autonomous changes within one of the systems should therefore entail changed »deformation tendencies» within the others. This renders it difficult or indeed impossible to establish the cause of the different chromosome-morphological changes observed.

8) The chromosomal spiral (*cf.* point 2) is the subject of a successive elimination, at least during the development from the early prophase to the late metaphase. This applies to mitosis as well as to meiosis. Despiralisation is not quite fulfilled — possibly with a few exceptions — when the effect of the anaphase forces becomes discernible. The chromosomes of the late metaphase therefore often show that their chromatids are slightly twisted around each other.

9) It is very likely that the despiralisation mentioned in point 8) started already during the anaphase of the preceding division or still earlier. If the latter be valid, the start occurred when the actual spiral was a chromatid spiral or a spiral of still lower order.

10) The length and diameter relation of the chromosomes may at least in part depend on their spiralisation conditions. The chromosomal spiral's elimination should in itself possess a chromosome-elongating and thinning effect. During the prophase-metaphase development, when

the chromosomal spiral is successively eliminated, chromosomal contraction, however, occurs. This should be due to a simultaneous »dominating» change in subordinate systems (*cf.* points 1 and 2).

11) The changes (mentioned in point 10) with a chromosome-contracting effect are possibly due to an increase in the degree of spiralisation of the chromatid spirals or to the chromatid spirals getting a new spiralisation pattern. Their previously straight or straighter longitudinal axis acquires possibly now itself a spiral shape (*cf.* point 5). This may occur without any real increase or even during a simultaneous decrease in the degree of spiralisation of the chromatid spirals. Changes in the degree of climbing, in the size of the diameter and in the number of turns of the major spiral also seem to contribute to these reactions. During early anaphase the still remaining attachment between the daughter chromosomes and the hemi-bivalents, respectively, together with the separating anaphase force brings about a temporary discontinuation of the process or even a certain degree of regression. This is compensated by a relatively rapid contraction as soon as the daughter-elements have released their mutual contact. The contraction then continues, reaching its maximum during late anaphase (or early telophase). Temporary elimination of the contraction or temporary regression can also be observed during metaphase in those cases where the chromosomes' distal parts show a more obstinate association with the poles of the spindle (*cf.* point 26).

12) The major spiral of the meiosis is, at least during a late stage, constituted of two mainly parallel-running chromatid spirals, not twined around each other. Consequently, during these stages the major spiral, considered as a unit, should be a so-called parallel spiral (paranemic spiral, anorthospiral). During earlier phases, however, the double spiral should be more or less a relational spiral (plectonemic spiral, orthospiral). This should be an expression of the still remaining chromosome spiralisation; the disappearance of this element should constitute the final phases in the elimination of the chromosome spiral.

13) All assessment of the spiralisation conditions and the underlying mechanism is particularly risky. The structures are so fine that they can only just be vaguely observed in the most favourable material. The description of the transformations must therefore be taken as merely an attempted interpretation. Consequently, all explanations in this field, including those given above, must be considered hypothetical.

14) The formation of karyomeres is a normal phenomenon. The »extrachromosomal» parts of the karyomeres seem to be identical with

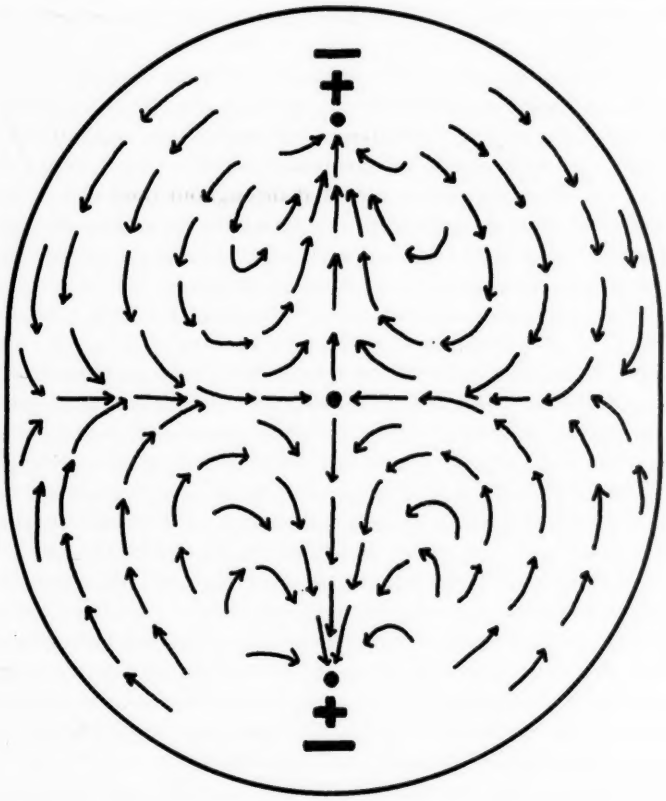

Fig. 1. Scheme approximately illustrating the movements of K^--poles placed free in a field of forces brought by dipolary placed, fixed double-poles of the following nature. The exterior partner in each double-pole is K^--charged and of a somewhat stronger power than its interior K^+-charged partner. The plus- and minus-signs symbolize the fixed poles, the arrows the direction into which the free K^--poles have to be moved. The arrow series demonstrate the running of the different force-lines in the K-field. The influence of the different free poles upon each other is omitted. The dots indicate places where the different forces of the field are equalizing each other.

the increased matrix substance of the anaphase chromosomes. The nucleus is formed more or less sluggishly by a fusion of the different karyomeres. Here it is possible that a surface tension process is involved. It is not excluded that the matrix of the chromosomes is to be

identified with a kind of matrix belonging to the chromatids inside the mother-nucleus.

15) During congression (prometaphase, metakinesis) and during anaphase the chromosomal movements (the centromeric movements) take place along lines lying at different distances from the central axis. Although the difference is inconsiderable, these lines during the congression are more peripherally located throughout their course; during the anaphase they are more centrally placed. This relationship applies to mitosis as well as to meiosis; in the latter case in principle both to bivalents and multivalents as well as to univalents.

16) The congression seems to occur along more or less curved lines. The removal goes primarily — perhaps even then in an arc — towards the more peripheral parts of the future metaphase plate or towards a circular line lying still more peripherally in the metaphase plane. After this there often follows a centrally directed movement in the equatorial plane. Possibly this is more or less often preceded by a delaying period. The secondary movement may possibly proceed relatively sluggishly.

17) Both the congression movement as well as this one together with the anaphase movement that follows and the latter alone can be taken to be a »summation movement». The reconstructed movement should occur if the following conditions were present:

a) The kinetochore of the centromere is under the influence of repulsion forces exerted by poles (particles, particle complexes, *etc.*) located in the central axis of the spindle as well as under an attracting influence of a pair of poles (particles, particle complexes, *etc.*) in the same axis but somewhat more centrally located, — *cf.* Fig. 1.

b) The size of the two repulsion forces that affect one and the same kinetochore and were mentioned in a) is reversed in proportion to the distance between the kinetochore and the poles in question. The same applies also to the two attraction forces.

Should the conditions now mentioned be present, we can then speak of kinetochore-attracting and -repelling poles (particles, centres, *etc.*). They are designated below by K^+- and K^--poles, respectively. Collectively, the abbreviations K-poles, K-forces, and so forth, are also used.

18) Several facts speak in favour of the centromeres (the kinetochores) at least normally repelling each other. They should do so if they themselves constituted a kind of K^--centre. If this interpretation and the ones in point 17) be correct, it is legitimate to speak of the centromeres as movable K^--poles placed in a more fixed field of K^+- and K^--forces in accordance with the scheme illustrated in Fig. 1.

19) If the interpretation given in point 17 be correct, the »primary» metaphase appearance with the centromeres in the periphery of the future metaphase plate or in a still more peripheral position is conditioned by the lines of force in which the different centromere kinetochores wander »colliding» here with similar lines belonging to the other half of the total field of force.

20) The permanent ring-shaped metaphase plates that characterize many organisms are perhaps due to the mutual repulsion of the centromeres, to the repulsion between these and extra-chromosomally K^--charged stuff eventually present and located in the centre of the metaphase plane, and to the inability of the centromeres to »push» this stuff away.

21) The at first ring-shaped metaphase nucleus' transformation to a »homogenous» metaphase plate is perhaps due to the ability of the chromosomes to »push» away the primary centrally placed extra-chromosomal K^--charged stuff, which was mentioned in point 20. The tendency for such a pushing away must arise, if the stuff in question consists of (relatively easily removable) particles with a lower K^--charging than that belonging to the centromeres.

22) When the centromere complex during mitosis first meets the equatorial plane it is said at least sometimes to be orientated so that the pair, which is formed by its two daughter-units, lies $\pm$ parallely with the above-mentioned plane. Oblique orientation and perpendicular orientation seem nevertheless to be present already during this stage. In spite of this variation, perpendicular orientation is always adopted when the equatorial plane has been reached. Should the attempted interpretation as given in point 17 be correct, a placing of the two daughter-units in the equatorial plane must entail a highly labile position of equilibrium. Oblique orientation as well as the least dislocation of the labile position just mentioned must induce the final perpendicular position.

23) The anaphase separation may be a secondary consequence of the perpendicular metaphase position of the centromere kinetochore. At the fulfilment of the said position the two daughter kinetochores come into each its half of the assumed field of K^--forces. Hence, each of them will be attached to the nearest pole. The force of repulsion acting between the daughter-kinetochores may act as a contributory factor.

24) The centromere scarcely always contains the only kinetochore of the normal chromosome. Particularly the distal or subdistal parts of especially long chromosomes at times reveal certain K^--characters. If this

be due to the normal chromosome in its entirety possessing such characters or that it here and there has particles of K^--character is uncertain. However it may be in this respect, the kinetochores in question seem to be considerably weaker than the centromere-bound ones. Whether it is a question here of a K-gradient that is sinking in a certain direction or of some other more or less regular system is uncertain.

25) The position of the different chromosomes and of their different parts in the metaphase plate — and during other nuclear phases — should be due to the relation between the size of their K^--charges, of the polar K-forces, of the general resistance offered to the preceding movements and of the positions during earlier phases.

26) If particularly long chromosomes are present, their distal or subdistal parts show a not infrequently more or less obstinate adherence to the poles during congression, during early metaphase and possibly also during late metaphase and early anaphase. This may be due to the K^--charge of the parts in question and to its relatively weak strength. These characters from the outset give to the sections just mentioned — *cf.* point 46 — a less pronounced peripheral placing in the K-field than do the centromeres. They should therefore from the beginning lie in lines of force — *cf* Fig. 1 — that run in more or less narrow arcs towards the nearest pole without having even touched the equatorial plane; or they have already earlier occupied such a position that they are already then attached to the poles.

27) The final orientation of the centromeres in the equatorial plane and the more or less similar placing of eventual subproximal kinetochores induce together with the chromosomes' elasticity a more or less pronounced tendency to a spreading out of each individual chromosome in the equatorial plane. This at the same time involves a tendency to eliminate the eventual polar attachment of the distal parts of the chromosomes. Consequently, a pair of force antagonists should be present here. The curving and stretching phenomena that are particularly shown by long chromosomes during the prophase-anaphase development, are in complete agreement with these elucidations.

28) The metaphase- and anaphase-spindle consists at least not only of single, evenly distributed »fibres» but at least in part of evenly distributed bundles of fibres. These or their equator-attached central parts seem on the one hand to repel each other, on the other to repel chromosomal elements in the spindle or its vicinity.

29) In addition to fibres and bundles running from pole to pole the spindle contains the bundles that run from the centromere to the poles

and from the daughter-centromere to the nearest pole, respectively. Each bundle is broadest in a region not very far from the centromere; it converges on its two end points.

30) The fibres that at least illusorily run from pole to pole are possibly, at least in part, composed of two halves »associated» with each other in the equatorial plane. At times there are pictures — bundles converging on the equator and poles — that support the view that the »seam» consists of invisible extra-chromosomal K^--particles.

31) The position of the chromosomes in relation to the spindle and to their separate fibres during the primary and secondary metaphase stage — *cf.* points 16 and 21 — speaks in favour of the centrally placed K^--charged, extra-chromosomal stuff, which may temporarily prevent the »immigration» of the chromosomes, being partly identical with the »seam-particles» mentioned in point 30.

32) All the more or less spindle-like or spindle-fibre-like structures that arise in a position other than that of the metaphase- and anaphase-spindles may in principle have the same background as that of the said spindles.

33) The view that all the spindles and the fibre systems mentioned in points 28—32 are the result of a sorting-out action (possibly first visible under the influence of the fixative) of the different K-charged complexes on the K-charged stuff in the neighbourhood should perhaps not be rejected out of hand.

34) The often fairly pronounced undulating course of the spindle-fibres, the occasionally occurring tail-like extension of both ends of the metaphase spindle as well as a number of other phenomena speak against the attempted explanation in the preceding point. The possibility that a number of »contradictions» are conditioned by the disturbing influence of the fixative can nevertheless not be excluded.

35) The occasionally stressed lack of accelerating anaphase movement, which is considered to speak against the view that electrostatic or »similar» forces are the cause of the anaphase movement, is explained if there really is a relatively strong K^--pole placed closely behind the K^+-pole (*cf.* point 17).

36) The RABL-field (the polar field), which belongs to the advanced anaphase nucleus, the telophase nucleus and also (at least during the early phase) the prophase nucleus, should be due to the monopolar-placed K^+- and K^--pole during the corresponding phases, to the forces of repulsion acting between the centromeres, and of course ultimately to the general K-force interplay then existing.

37) The widening of the RABL-field occurring during prophase may be due to the then existing intercentromeric repulsion having increased in relation to the kinetochore-attracting influence of the polar complex.

38) The prophase stage with an even distribution of the centromeres along the whole periphery of the nucleus — if such extreme conditions really arise during mitosis (*cf.* point 72) — involves an intensifying of the conditions mentioned in the preceding point.

39) Prophase is terminated — at least often — by a more or less pronounced contraction of the nucleus.

40) The contraction nucleus, mentioned in the preceding point, scarcely permits a morphological analysis. The material is too strongly concentrated. Nevertheless, the chromosomes seem to be strangely curved. The ends in extreme cases give the impression of having fairly central positions. The centromeres give a similar impression, although to a considerably smaller degree. The entire chromosomal mass gives a more or less dorsiventral, at times a fairly lobed impression. The spindle seems to be represented by a number of fibrous units or fibre-bundles, running in different directions, at times intersecting each other in different planes, and often converging in different directions.

41) The background for the establishment of the contraction nucleus may be the following: The relation between the strength of the centromerically placed kinetochores and that of those more distally placed gives to the former during prophase a »more strongly polarised» position than to the latter. The K^--centre, which was hitherto a single one, is divided immediately before the formation of the contraction nucleus. The daughter poles force each other into a mutually opposing position. This results in the hitherto monopolar-placed, undivided K^+-centre being given a central position. The transformation of the poles proceeds relatively quickly. The chromosomes are less mobile. The RABL-field, which nevertheless clearly undergoes a certain disturbance, will therefore temporarily remain unchanged. The K^+-centre comes nearer to the distal kinetochores. On account of the distances these, in spite of their weaker K-characters, are affected more by the K^+-centre than by the centromeres.

42) After the contraction there sometimes follows a nucleus shaped like a sheaf (»Garben-Kern»). These are likewise difficult or impossible to analyse morphologically. Here also, however, curious chromosomal curves are present. The distal parts of the arms give the impression of being attached at random to the two poles. The centromeres give the impression of being more or less clearly centrally placed but at the

same time of being diffusely divided up into two groups. The front between these should coincide with the future equatorial plane.

43) The background for the transformation of the contraction-nucleus to the »sheaf-nucleus» may be the following:

The centrally placed K^+-centre is divided. The daughter products are moved closer to the K^--poles. The more distal-placed kinetochores that have already been brought closer to the mother centre will be attached at random to the different K^+-daughters and take part in their wanderings. In spite of their greater K^--charging, the centromeres, on account of the existing distances, show similar reactions only to a considerably lesser extent.

44) The »sheaf-nucleus» — as well as the contraction-nucleus not followed by a »sheaf-phase» — is not always directly succeeded by the real congression. (This impression is actually based not as much on mitotic as on meiotic studies.) An intermediate stage, during which the centromeres deviate more comprehensively from each other, would appear to be present.

45) The background for the »distending» mentioned in point 44 may be the following: the K^+-daughter poles — *cf.* point 43 — are displaced relatively quickly towards the poles. The distance between these and the centromeres is hereby increased. On account of the lesser mobility of the chromosomes the distance is hereby increased between the centromeres and the K^+-poles. This gives to the forces of repulsion obtaining between the different centromeres an ascendency over the newly prevailing endeavour of the undivided K^+-pole to bring them close to each other.

46) The contraction- and the »sheaf»-nuclei create entirely different pre-requisites for the subsequent movements of the centromeric and the distally-bound kinetochores. At least where chromosome-arms of over a certain minimum length are involved the centromeres and the kinetochores last-mentioned will lie within entirely different parts of the K-field at the outset of congression — *cf.* point 26.

47) The mitotic interplay hitherto described seems to be due to all the different K-charged units getting a stronger or weaker absolute and relative effect together with different placing during the different phases. This interplay would also appear to be connected with their ability to reproduce by division and their ability to increase in growth, with the fact that these processes do not occur simultaneously and that the moving of the different units does not take place at one and the same speed. That each daughter unit that has arisen by division of a

mother unit is at first in possession of a lower K-charge than the mother unit itself and that the daughter unit's charge then grows in such a manner as to develop into a new mother unit seems almost to be an axiom.

48) According to the summary hitherto presented the »maximally complicated» mitosis should show the following phases: a) prophase with a more or less strong concentration of the RABL-field, b) contraction-nucleus, c) »sheaf»-nucleus, d) more distended nucleus, e) congression, f) primary metaphase stage, g) secondary metaphase stage, h) anaphase, i) telophase. Numerous abbreviated patterns, however, doubtless arise in many organisms. On the other hand, one or more of the phases are sometimes lacking in an organism or in a tissue that also show a more complete pattern. The prophase nucleus with an even distribution of the centromeres along the periphery is perhaps the phase most generally lacking. The contraction- and above all the »sheaf»-nucleus are perhaps also wanting in many cases. Should all these phases and in addition the congression have been »jumped over», the RABL-field itself — eventually after enlargement and an insignificant re-orientation — will function as a metaphase plate.

49) Whether mitosis proceeds in a complete or reduced form may be determined by the speed at which the different underlying processes take place. Should the underlying interplay proceed so swiftly that one or more chromosomal equilibrium positions, otherwise arising, are not reached in time, the impression must of course be that one or more phases have been omitted.

50) Extremely polytene chromosomes, chromosomes of abnormal size, and so on, in certain nuclei in organisms that otherwise show normal conditions speak in favour of the opinion that the reproduction of the chromosome-building threads and the fulfilment of the different mitotic phases do not — at least not always — occur in accordance with a definite synchrone schedule.

51) The so-called C-mitosis seems to be conditioned by a »poisoning» of the polar complex or of certain parts of it. Especially the all-sided radially built chromosomal assembly described in connection with C-mitosis, speaks in favour of the K^+-pole having increased its influence in relation to the K^--pole. If this be really due to a direct increase of the former's strength or to the latter's total or partial disintegration escapes assessment.

52) The chromatids in the so-called C-pairs temporarily hold together, at least illusorily, only in the most proximal section of their

arms (the »LIMA-effect»). The background for this is difficult to assess. The circumstance may, however, be connected with the fact that the earlier association of the chromatids has been eliminated without the intervention of the polar-conditioned anaphase forces. It is possible that the elimination of the chromosomal spiral has forced a compulsory twisting within the centromere region, which in its turn temporarily prevents the proximal separation. — *Cf.* point 90.

53) If the proximal attachment points mentioned in point 52 are due to compulsory twisting within the centromere region, their gradual disappearance should be able to be traced back to a secondary autonomous elimination of the same.

54) The members, released from each other, of one and the same C-pair show, as is known, a distance-association that remains for a relatively long time. When the members are still held together, the mutual position of the different pairs is possibly conditioned by the interplay between their K-forces and those of their surroundings. One or other of the pair's replacement by the units in them should then probably not cancel the existing equilibrium but make it labile. The sluggishness of the system should make the conquest of a new, stable position of equilibrium — *i. e.*, the cancellation of all remaining distance pairing — only cease quite slowly.

55) More vigorous synchronism variations than normal seem to be the background for the so-called endomitoses and the formation of so-called polysomatic nuclei. The reproduction within the complex constituted by the chromosome together with the gradual and successive elimination of the actual chromosomal spirals have obviously been more comprehensive than what normally corresponds to the number of mitoses fulfilled. In certain cases the synchronism disturbance may be due to a precocious cessation of the nuclear division.

56) The C-pair-like structures arising in connection with polysomaty should have the same background as the real C-pairs.

57) The complex chromosomes described in connection with polysomaty that are characterised by a gathering of chromosome-arms »streaming out» from a common centromere region may be due to the reproduction of the one C-pair-like structure not being completed when the daughter structures themselves are starting their division. The degree of complication depends on the number of »collisions».

58) Syndesis occurs during the meiotic phase between more or less slightly spiralised but nevertheless spiralised or at least one-dimensionally twisted chromosomes differentiated into chromatids.

59) The bouquet-stage of the meiotic prophase can probably be due to all the kinetochores of the centromeres as well as all other not too weakly charged kinetochores having been attracted by the K-pole complex. The not pole-attracted, intermediate sections form the chromosomal arcs that are characteristic for the stage. (The reports based especially on zoological material that the centromeres as distinct from the distal poles of the chromosomes do not show the polarisation mentioned, speak nevertheless aginst this interpretation.)

60) The presumably as good as generally accepted view that local syndesis cannot occur between more than two homologues seems not to be quite convincing. (*Cf.* points 65 & 67.)

61) The circumstances mentioned in point 60 eliminate certain objections that previously could be made against FRIEDRICH-FREKSA's chromosome model. According to this, syndesis was conditioned by dipoles placed perpendicularly to the longitudinal axis of the chromosomes. Homology should imply that dipolar systems of different strength and composition were placed out in accordance with conforming patterns.

62) Should the bouquet stage be conditioned in accordance with the explanation in point 59, the kinetochore position then obtaining must facilitate the syndesis that follows soon after. The kinetochores as well as the chromosomal regions lying near them are compelled by the K-pole complex to move so close to each other that the syndetic force is enabled to make itself felt. If the syndetic force be conditioned in accordance with FRIEDRICH-FREKSA's conception, it can of course assert itself at the simultaneous presence of a mutual kinetochore repulsion that dominates it only when the latter is overcome by the kinetochore-attracting force of the polar complex. The secondary extinguishing of the syndetic forces can of course be due, among other things, to an increase in the mutual kinetochore repulsion, to a diminishing of the polar attraction or to a combination of these factors.

63) There is perhaps a connection between the spiralisation conditions of the chromosomes and their syndetic ability. More strongly spiralised chromosomes or chromosomes with more closed spirals perhaps repel each other, even if they are homologous. On the other hand, unspiralised, slightly spiralised and more drawn-out, homologously built spirals perhaps possess syndetic ability.

64) FRIEDRICH-FREKSA's chromosome model seems to be capable of explaining the supposed connection in point 63. A closer spiral creates an outwardly neutral structure. The kinetochore-conditioned forces of

repulsion then step into the foreground. At low spiralisation the attraction between the perpendicularly placed dipoles perhaps overwhelms the efforts of repulsion just mentioned.

65) The spiral twist of the syndetic partners retards the completion of the syndetic process or renders it more difficult. The thread system's primary mutual moving towards each other (hemisyndesis) can only be followed by final association (holosyndesis), if the syndetic force be so great that it is able to compel the partners to such transformations of shape that they fit into each other. The completion of syndesis must then imply that the partners are at least partially twined round each other; that a »bivalent spiral» is formed.

66) To bring about the secondary process mentioned in point 65 we require, as already mentioned, a compulsory deformation of one or both of the partners. The adaptation of two threads to each other must be a relatively simple matter. Mutual adaptation of three or more threads is however a very complicated process; possibly there is no time for it to be accomplished before the moment when the syndetic forces are eliminated.

67) The circumstances referred to in point 66 should furnish the reason why local syndesis is never completed between more than two partners. This should be the background for the view, mentioned in point 60, which in my opinion is not quite sufficiently founded.

68) The ultimate cause of crossingover and chiasma formation should be that each chromosome possesses two sides capable of syndesis. Should different side-pairs be exploited in two different sections, a chiasma-like contact point between two non-sister chromatids is in certain combinations formed at the final exchange point. This contact point is dissolved without genetic consequences or undergoes crossingover, at which, entirely automatically, the formation of a real chiasma follows.

69) The twisting of each of the syndetic partners constitutes the most important pre-requisite for the changes in the syndetic pattern (*cf.* point 68) and consequently also for crossingover and chiasma formation.

70) The interchiasma »opening out» during diplotene seems to be due to several factors. The most important of these seem to me to be a) a real repulsion following upon the extinguishing of the syndetic forces, and b) tensions caused by the spiralisation of the chromosomes and their chromatids.

71) Chiasma terminalisation seems (when it is a reality) to be due to several factors. The most important of these seem to me to be the forces

mentioned in point 70 a, as well as the successive elimination of the intertwisting of the chromatids.

72) The chromosomal position inside the diakinese nucleus can be designated as equivalent to a RABL-field, almost entirely spread out over the whole periphery of the nucleus. — *Cf.* points 37—38.

73) The attachment between the chromosomes that build up a chiasmafree bivalent, is perhaps due to several factors. In addition to a common matrix we must reckon with the consequences of the position of equilibrium previously obtaining. On account of the general balance obtaining until then, two units, just released from each other, only quite slowly change their mutual position — *cf.* point 54. Distance-association between homologous univalents previously constituting syndetic pairs, and between bivalents that have previously formed multivalents, should share the same background.

74) Contraction- and »sheaf»-nuclei — *cf.* points 37—43 — are, at least often, comprised as normal features of the first meiotic division. These nuclei can scarcely, or indeed not at all, be morphologically analysed. If the bivalents be short, the »sheaf-nucleus» demonstrates a general but mainly bipolar-directed »expulsion» of the chromosomes.

75) The bivalents conquer the metaphase position in principle in the same way and in principle for the same reason as the chromosomes during mitosis.

76) The univalent can show different patterns of behaviour, namely: a) It wanders more or less retarded into the equatorial plane where it is divided. A hemiunivalent wanders towards each pole. b) It does not reach the equator. As a rule undivided, it is then incorporated into one of the daughter nuclei. c) It reacts at first as in b) but remains lying outside both the normal daughter nuclei. d) It reacts at first as in b) or c) but shows during the late phase — when it lies in the spindle — a more or less clear tendency to division.

77) The fairly generally accepted view that the univalent's fate is determined by the degree of autonomous development that its centromere has reached lacks weighty reasons. As justified is the view that the autonomous development now mentioned lacks significance here; that the conquest of the equatorial position is something primary, that the division is the secondary effect of this conquest.

78) The univalents seem just like the bivalents to wander in arc-shaped lines. This is entirely explained, if the K^+- and K^--poles exert the influence on the kinetochores as postulated. Univalents, which are incorporated in the equator, lie in lines of force running into the equa-

torial plane during the phase of development when the incorporation takes place.

79) The univalents, later incorporated in the equator, show a temporary, fairly obstinate slackening in their congression movement. They are localized in the equatorial plane peripherally around the bivalents that have already wandered in. The phenomena appear entirely natural if the attempts at interpretation in points 17—21 be correct. The univalent's centromere complex possesses only half as great a K^--charge as that of the bivalents. Hence, the bivalent has a considerably greater possibility than the univalent to »compete» successfully with the extrachromosomal K^--charged substance, which shows a greater or lesser tendency to an early, central accumulation.

80) The univalent, incorporated in the equatorial plane, is divided later than the bivalents, often not until the »latters'» anaphase. When during the bivalent anaphase the bivalents disappear from the equator, the univalent moves closer to the centre, really close to which it nevertheless never seems to come. This appears to be the natural consequence of the conditions mentioned in points 17—21.

81) The univalents not reaching the equatorial plane wander, at least in some cases, in arc-curved lines. In their first part these are central-directed; finally, however, they run out into the nearest pole. The occurrence is supposed to be general. The curving degree of these lines should be of different strength in different cases. The background may be the following: On account of its low K^--charge the univalent often lies in or is often forced into those parts of the K-field where the lines of force form arcs that are more or less close to but do not reach the equatorial plane and that run into the nearest pole. It does not seem improbable that a greater or lesser number of the univalents not reaching the equator have primarily belonged to lines of force that have had contact with the equator. The protracted slackening — *cf.* point 79 — makes possible however, such a re-shaping of the field of force that this contact has been secondarily lost. The pre-requisite should here be an autonomous change of the relative force of the K^+-poles or a change of their mutual distance.

82) The behaviour of univalents that have never been incorporated in any of the regular daughter nuclei or that »cause» the formation of a restitution nucleus may be conditioned by an extreme prolongation of the slowing up characteristic of the univalent congression.

83) The tendency to a late division of a more or less strongly slowed-down univalent should be due to its autonomous development and to

the repulsion acting between its daughter-centromeres. If the tendency appears when the univalent lies quite near the equatorial plane, the result can be a real division and a distribution of the hemiunivalents to the different poles. In other cases the entire univalent »in the act of division» is incorporated in the nearest of the two ordinary daughter nuclei or remains lying outside but more or less close to it.

84) The similarity in behaviour shown by the univalents during meiosis, by the distal parts of long chromosome arms and acentric chromosome fragments is striking. The differences should be due to the relative strength of the kinetochores of the different units. This determines where the said units is placed in the K-field and also to what degree it is slowed down by the extra chromosomal K^--charged substance.

85) The mutual position of the different units in the equatorial plane during the heterotypic metaphase is determined by the relation between their size and their K^--charge, their earlier position and the resistance they have offered to the foregoing changes — *cf.* point 25.

86) Single centromeres, pairs and complexes of centromeres are orientated in different manners during the congression of the heterotypic division. Bivalents of ordinary shape show upon the whole the same characters as chromosomes during the corresponding mitotic phase. Perpendicular orientation already before the reaching of the equator is on the other hand particularly common. Already during this early phase more or less perpendicular-oriented bivalents with centromeres vigorously departing from each other are common, when the latter are situated more remote from the chiasma or chiasmata present. This should be due to a different influence of the pole-bound forces upon the different centromeres and upon the effect of the repulsion existing between them. During the metaphase there is in each case a very fixed orientation. A labile balancing of one or more centromeres belonging to one and the same unit is never realised. The different position's degree of stability is due to the degree of intimity in the attachment between the different elementary units of the centromere, the pairs of centromeres and the complex of centromeres, respectively. A zigzag orientation is the consequence if all the internal distances be more equal and of such an order of size that they lie between a certain maximum and minimum value. The distribution of the centromeres upon the different poles is reductional — here the bivalent only is concerned — if the centromeres of the sister-chromatids are held together more closely than those of the non-sister-chromatids. All other existing

patterns of orientation may possibly be explained in a similar manner.

87) The conditions mentioned in point 86 compel the trivalent, the pentavalent, and so on, to place — with the exception of cases where one of the centromeres balances in the equatorial plane — one centromere more on one side than on the other of the equatorial plane. The interplay of forces then entails almost immediately that the multivalent in question (at the same time as it is worked upon in a dividing direction) is moved more towards the pole that receives the centromeric-surplus than towards the other (the »ÖSTERGREN-effect»).

88) The non-random or in extreme cases entirely one-sided distribution of the non-equator-entering univalents that at least in many univalent-bearing *Rosa*-biotypes arises during the EMC-development is possibly due to the general heteropolarity present here. This should also imply that the two polar K-complexes of the metaphase and the anaphase nucleus are of different strength.

89) As a heteropolar division is in no way something unique for *Rosa*, the univalent distribution during the EMC-meiosis should be investigated more in detail in different organisms.

90) The peculiar type of attachment (the »LIMA-effect») that characterizes the two chromatids of the chromosome during the anaphase and telophase of heterotypic division as well as during the following prophase may have the same cause as the similar conditions inside the so-called C-pairs during their attachment-phase — *cf.* point 52.

91) »Contraction nuclei» can be met during the homeotypic division. These or rapid processes »corresponding to them» in the underlying mechanism (*cf.* points 48—49) result — at least when it is a question of very long chromosomes — in the distal or subdistal parts of the chromatids being attached to the poles. The attachment occurs at random. Two chromatid-arms corresponding to one and the same chromosome-arm can here be bound to different poles.

92) The metaphase and the anaphase of the homeotypic division agree in their development in principle with the corresponding mitotic stages. There arises, however, a complication, namely that the »LIMA-effect» is eliminated. Parallely with the forcing of the chromatid into the equatorial plane, the proximal attachment between the two members of a chromosome is »stretched out» over an increasingly larger part of each of its two arms. The phenomenon is often followed by the chromatid-arms corresponding to one and the same chromosome-arm being twined around each other. The same occurrence takes place when the actual chromatid-arms were distally attached at one and the same pole and

because of this compelled to stretch owing to the equatorial incorporation of the corresponding centromere. When the actual chromatid-arms were more permanently attached to different poles a »terminalisation» of the earlier proximal point of attachment occurs during the anaphase. This »terminalisation» often takes place during a mutual twisting of the partners. These conditions constitute a certain support for the assumptions put forward in points 52, 53 and 90.

93) Hemiunivalents, eventually existing during the homeotypic division, behave in principle in the same way as the univalents during the first division. The cause should be the relative strength of their K^--charges.

94) Inside dyads and tetrads still lacking partition-walls, in bi- and tetravalent coeno-macrospores as well as inside immature embryosacs and endosperms without partition-walls, and so forth, the mutual position of nuclei, still capable of division, and their poles is possibly due to the repulsion caused by the existing K-forces. The changed nuclear position after an eventual formation of partition-walls should be due to the »isolating» effect of the latter.

95) The so-called BAMBACIONI-effect, characteristic of the development of many tetrasporic embryo-sacs, should at least partly be due to the compressing influence exerted by the K-komplex of the dominating, apical-placed nucleus. The weak development of the basal nuclei seems to be directly correlated with a lowering of the repulsion effect acting between them.

96) The multipolar spindles at times appearing after the formation of a restitution nucleus and in certain other cases may be due to the presence of supernumerary K-poles.

97) The »contraction nuclei», which cause an »elimination» of the chromosomal number's reduction during the EMC-meiosis of many agamosperms, may be conditioned by disturbances in the developmental cycle of the K-complex. The many variations arising are possibly conditioned by the intensity of the said disturbances and the moment for their intervention. These disturbances and those inducing the polysomatic conditions — *cf.* point 55 —, seem to be of one and the same nature.

* *
*

As has already been repeatedly pointed out above, many of the attempts at interpretation given now must be considered as hypothetical in a high degree. The conception that the meiotic and mitotic interplay are conditioned by what has been called the K-forces was from the outset based entirely on the chromosomal wandering during the congression and anaphase of the mitosis. As demonstrated above the actual conception has shown itself also capable of explaining — without recourse to auxiliary hypotheses — the reactions of the univalents, the centromeres' metaphase orientation in different cases and the chromosomal position during many different phases of mitosis and meiosis. Of course this fact does not constitute any really convincing indirect proof of the accuracy of the conception but it does lend it a certain support. If the K-forces be present, we cannot entirely exclude the possibility of their being of a quite special kind; that it is a question of what have been called »mitokinetic forces». It should however be noted that the reactions described are such as would result, if a purely electro-static system were involved. Here, experimental control is highly desirable. The possibility that in all circumstances it is a question of a kind of »remote-action» is, however, considerable. The author unfortunately lacks the necessary knowledge to assess whether a similar effect can be induced by changes in the medium lying between the, at least illusorily, more directly reacting units. Simple currents or elastic fibres of a more or less concrete kind can however scarcely explain the interplay.

EFFECTS OF GIBBERELLIC ACID ON ERECTOIDES MUTATIONS IN BARLEY

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INTRODUCTION

BY treating seeds of barley with mutagenic agents different kinds of mutations can be induced, amongst which the erectoides type is very common (GUSTAFSSON, 1947). The most conspicuous feature of this mutation is the dense ear, but usually also the straw is shorter and stiffer than in normal plants. The reason for the dense ear can be traced back to a shortening of the internodes in the rachis. Up to now 70 different erectoides mutations, which are distributed over at least 22 loci, have been genetically investigated (HAGBERG, 1958). Each mutation behaves as a single segregating factor. The occurrence of so many mutation loci indicates that in normal plants a rather complex biochemical and physiological sequence is operating, which obviously can be blocked or changed at many points by a mutation. Very little is known about the biochemical background of these mutations, but there are good reasons to suspect that the biosynthesis or action of one or several growth promoting substances may be inhibited. As to the nature of possible substances it seems reasonable to guess on especially those types which are known to increase internode lengths in plant stems in general.

Such a class of substances are the gibberellins, as has been shown by many authors (YABUTA and HAYASHI, 1939; BRIAN *et al.*, 1954; FEUCHT and WATSON, 1958). Especially interesting in this connection are the reports on the role of gibberellins or gibberellinlike substances in genetic dwarfs of *Pisum* (BRIAN and HEMMING, 1955) and *Zea mays* (PHINNEY, 1956). In these dwarfs normal growth can be induced by adding gibberellic acid to the plants, thereby indicating that the synthesis of gibberellinlike substances is blocked. If a similar system were working in the erectoides mutations it should be possible to restore the plants to normal appearance by supplying them with a small amount of gib-

berellin. This paper is a short communication on some preliminary experiments to test this hypothesis. Application of gibberellins to compactoid-forms of *Triticum* has not given any significant results (WRIGHT, 1958).

MATERIAL AND METHODS

The experiments have been performed with the variety Bonus and six erectoides mutants obtained from this variety. The following mutation types were used: *ert-a*[28], *ert-m*[40], *Ert-r*[52], *ert-c*[47], *ert-d*[80 h] and *ert-u*[56], (HAGBERG, 1958). The plants have been cultivated in normal soil both in the field and in the greenhouse, in the latter case however only Bonus and the 4 first mentioned of the mutants have been tested.

Greenhouse experiments. The material was sown on March 22nd in pots, six seeds in each. Before treating the plants the four best ones were selected. Two of them were taken as controls and not treated, to the others gibberellic acid (GA_3) was given at different dates and in different ways. Most of the material was harvested on July 16th.

Field experiments. The plants were sown on May 13th, treated once with GA_3 (June 30th, 2 μg in 10 μl alcohol applied on the top leaf of each shoot) and harvested on August 26th. No ears had emerged at the time of treatment. The oldest shoots were in the stage of meiosis (25 cm high), the youngest ones about 15 cm high.

After harvest the length of the straw and the ear and the length of the internodes of the stem and the ear were measured. Unfortunately some of the labels on the single shoots of the plants from the field material were destroyed, so the sequence of the registered shoots may be somewhat inadequate here and there, but the error introduced in this way can only be of minor significance, as the results obtained by visually arranging the shoots in falling series after their size, very well fit a general picture.

RESULTS AND DISCUSSION

Greenhouse material. These experiments served mainly to find out the most suitable way to apply the substance and the right stage of development of the plant for the application. Concerning the first question a method had to be used which was available both in the greenhouse and in the field without any risk of contaminating the control plants. Spraying the material with a solution of the chemical therefore had to be avoided. Two other methods were tested, namely to

TABLE 1. *Response of erectoides mutants and mother type to treatment with gibberellic acid.*

(Treatment: one single application after 56 days of 2 μg GA_3 in 10 μl alcohol to the top leaf. The internode length of the ear is expressed as the length of the next 10 internodes after the 5th fertile flower. All lengths in mm.)

Material	Total length of straw		Length of ear		Internode length of ear		
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Difference %
Bonus	870	1050	80	97	32	45	+ 41
	850	1040	80	95	32	45	
ert-a[23]	940	1050	67	80	24	32	+ 36
	910	1080	64	80	23	32	
Ert-r[52]	720	700	47	50	19	18	— 5
	750	720	51	51	20	19	
ert-c[47]	710	825	64	82	28	36	+ 28
	725	870	76	78	29	37	

ert-m[40] was damaged and therefore excluded.

apply drops of the solution to the top leaves or to inject the solution into the stem. Both procedures proved to be effective, the first one perhaps being somewhat better, and since it was also easier to carry out, it was preferred in later work. Both water and 95 per cent alcohol were tested as solvents, of which the latter gave better results owing to its rapid penetration into the leaf.

The time of application proved to be of great importance for the action of gibberellic acid on the internode length of the ear. Early application increased the length of the lower internodes in the stem, but the effect was not persisting long enough to have any influence on the development of the ear. On the other hand treatments later than the stage of meiosis had no effect either. The most sensitive stage therefore seems to exist about a week prior to meiosis, *i. e.* at the moment when the plant changes from the vegetative to the generative phase. According to a recent article (KAUFMANN and TWEEDIE, 1958) stem elongation in rice is most sensitive to added gibberellin at just that moment.

The results from a treatment in the most sensitive stage are shown in Table 1. It is remarkable that not all types reacted in the same way, only two of the mutations gave a positive response, whereas the third one was completely unaffected. As will be shown further on, the field

TABLE 2. *Effect of gibberellic acid on different developmental stages of erectoides mutant ert-a*[39].

(Treatment: one single application after 48 days of 2 μg GA_8 in 10 μl alcohol to the top leaf of each shoot. The numbering of the internodes begins from the top, the numbering of the shoots from the oldest ones.)

Straw number	Length of straw	Internode length of the stem					Length of ear	Internode length of ear
		1	2	3	4	5		
Untreated								
1	530	175	140	90	90		68	25
2	505	130	130	80	95	55	68	24
3	490	150	130	85	85		61	25
4	490	160	130	80	90		64	25
5	470	190	95	80	80		55	22
Treated								
1	540	265	130	65	50		83	28
2	550	200	150	80	55	50	85	28
3	500	185	160	95	50		101	38
4	540	165	120	115	85	55	82	33
5	540	210	115	110	80		71	28
6	490	120	100	85	100	65	62	25

experiments gave similar results and the phenomenon will be shortly discussed in connection with them.

Field material. A typical result from the application of GA_8 to a sensitive erectoides mutant is shown in Table 2. Here it can be seen that the internode length of the ears in the untreated plant has a very constant value, whereas there exists a great variation in the treated material. Some of the treated shoots bear ears with rather long internodes, and as a matter of fact they may even be longer than in the normal Bonus type, but others have reacted very little (Fig. 1). The reason is of course that the shoots were in different stages of development at the time of application, which was shown previously to be of great importance in the greenhouse experiments. Obviously the oldest shoots had already passed their most sensitive stage (in respect to the effect on ear density), while the youngest ones had not reached it yet. This result strongly supports the assumption of WRIGHT (*l. c.*) that his negative results with compactoid wheats may be due to incorrect time of treatment. In Fig. 1 some ears from a plant treated with gibberellic acid are compared with a set of ears representing the variation in ear density caused by mutations. It is of interest to note the similar phenotypic effects produced by both »modification» and »mutation».

Fig. 1. Different ear densities obtained as induced mutations (1—4) and by treatment of the mutant *ert-a*^{28} with gibberellic acid (5—8). 1=quadruple recessive (*aa*, *bb*, *cc* and *dd*). 2=*ert-a*^{28}. 3=Bonus mother strain. 4=*lax-3*. 5—8=*ert-a*^{28} treated with GA_3 at different developmental stages.

TABLE 3. *Response of erectoides mutants and mother type to treatment with gibberellic acid (Field material).*

(Same treatment as in Table 2.)

Material	Total length of straw		Length of ear		Internode length of ear		
	Untreated	Treated	Untreated	Treated	Untreated	Treated	Difference %
Bonus	550	625	99	125	37	54	+ 46
ert-a^{23}	495	500	63	101	24	38	+ 59
ert-m^{40}	355	525	86	109	30	40	+ 33
Ert-r^{52}	420	485	63	69	21	26	+ 24
ert-c^{47}	410	525	68	89	28	39	+ 39
ert-d^{30h}	380	460	57	65	20	23	+ 15
ert-u^{56}	385	320	101	89	32	43	+ 35

In Table 3 some characteristic data from treated and untreated plants of each mutant are collected. A close inspection of this table reveals some interesting facts. The response of the internode length in the ear to the treatment with GA_3 is positive in all cases, but in a highly varying degree. The maximum and minimum values are 59 and 15 per cent respectively. Already in the greenhouse material it was noticed that not all mutants responded in the same way to treatment with GA_3 and this is not surprising since the mutations are located in different loci and therefore can be expected to act on different sites in the biochemical and physiological reaction chain. Such an interpretation may be supported by a parallel case in *Zea*, where it has been shown that gibberellinlike factors have differential biological properties in certain genetic dwarfs (PHINNEY and NEELY, 1958).

It is evident from the table that parallel to the increase of internode length in the ear there is an increase also of the length of the ear and the straw in all cases but one, *e. g. ert-u*^{56}. This mutant shows a strong, positive response of the internode length in the ear, but a slightly negative one of the ear and stem length. The material is not large enough to permit any definite conclusions, but it is tempting to speculate if there may be differences in the action of gibberellic acid on cell elongation and cell division in the investigated mutants. Further work with larger material is needed for a definite answer.

Finally it should be pointed out that the normal type responds as strongly to treatment with gibberellic acid as the most responding mutants. A possible interpretation of this fact seems to be that in the normal plant there exists a certain balance between growth promoting

and growth inhibiting substances. This balance can be altered by mutations as well as by application of proper chemicals. The mutations ordinarily cause a shortening of the internodes in the ear but there also exist some cases where the ears have become more lax than in the normal type. So far only a lengthening of the internodes has been achieved with chemicals, but by treating a normal plant with suitable growth inhibitors it may also be possible to get a »dense ear»-modification.

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SPARSELY CONTRA DENSELY IONIZING RADIATIONS AND THE ORIGIN OF ERECTOID MUTATIONS IN BARLEY

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OWING to their characteristic phenotypes, their agricultural importance and the great number of corresponding loci the erectoides mutants in barley have attracted a wide interest in recent years. It is not our intention here to summarize all the analyses published. It will suffice to refer to the papers cited in the literature list. The chief characteristics of the erectoid mutants are as follows:

The morphological changes are in general striking and pleiotropic, affecting various ear and straw properties. For this reason the mutants are easily recognized and isolated, also in large offspring with low mutation rates. Similarly their physiological reaction is characteristic. Often the mutants make better use of a high nitrogen dressing than their mother strains do. They imply a sort of »nitrogen ecotypes».

Some mutants are high-yielding in field trials. One of them has been approved as an »Original variety»: Svalöf's Pallas barley, and others undergo large-scale testing. High-yielding erectoids are formed by mutation in numerous gene loci.

After diallelic crossings, tests of allelism and linkage (including chromosome arms marked with translocations) up to 69 (70) individual cases of mutation have so far been referred to 22 different loci (Table 1). The phenotypical appearance of the individual cases of mutation is studied continually and their productive capacity tested in collaboration with the Barley Department of the Swedish Seed Association at Svalöf.

In 1947 studies were begun in order to analyse possible differences in mutagenesis caused by densely ionizing radiations like neutrons *contra* sparsely ionizing ones like X-rays. It was reported by GUSTAFSSON and MAC KEY (1948), as well as later by MAC KEY (1951, 1952), EHRENBERG

TABLE 1. *Erectoides mutations and their locus distribution.*

(γ, X, p, n, α denote mutations induced by γ- and X-rays, protons, neutrons and radon α-rays respectively. T denotes the occurrence of chromosome breakage at the erectoid locus)

Locus	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*	*m*	*n*	*o*	*p*	*q*	*r*	*s*	*t*	*u*	*v*
Mutations	6X	2X	1X—T	7X—T	17X	18X	24X	25X	27X	31X	32X	12X	34n	51X	16X	44X	45α	52n	50X	55X	56X	57n
	11X	4X	14X—T[1]	14X[1]	65p		80X		53n				35n				72n	67X				
	13X	5X	39X	15X			84n						40X									
	19X	9X	47n—T	30X[3]			90n						41X									
	21X		48X	30X[3]									42X									
	23X		59n—T	33n									54n									
	28X		62n	43X									64n									
	29X		66n	60n																		
	36X		70n—T	89γ																		
	38X		75γ—T																			
	49X		82n[2]																			
	77γ		83n[2]																			
			85p[2]																			
			88γ[2]																			

[1] In the case of erectoides 14 two loci mutated simultaneously. A translocation is present, having one point of breakage at locus *c*.

[2] These erectoids are not yet analysed with regard to chromosome translocation.

[3] Two different alleles were isolated out of the same spike progeny.

et al. (1952) and EHRENBERG and NYBOM (1954) that the effects of the two groups of radiation turned out to be different, both with regard to physiological properties such as seed germination and seedling growth and genetical properties such as induced sterility. A feature, now being examined in a more precise way, was the high incidence of erectoid mutations, relative to other viable mutation types, in materials treated with fast and thermal neutrons (*cf.* also NYBOM, 1956).

EHRENBERG *et al.* (1956), as well as HAGBERG (1958) and BORG *et al.* (1958), have drawn the conclusion that there are different mechanisms of mutation acting in separate loci, when different kinds of radiations are applied. This problem has been further pursued in this article. In addition to X-rays, agents like chronic and acute γ-irradiations from cobalt-60 have been used, likewise fast and thermal neutrons produced in cyclotrons or reactors, accelerated protons from the synchrocyclotron and α-particles from radon or polonium. The different sources of radiation and their physical properties have been discussed by EHRENBERG (1954).

In Table 1 the 69 (70) localized mutations are arranged according to locus order. In previous papers (for instance HAGBERG, 1954; GUSTAFSSON, 1954) the *a* locus was considered the most frequently mutating one, but gradually, as the neutron data gathered, more and more mutations belonging to locus *c* were discovered.

Some mutants are not very useful in allelism tests. This is the case with ert-12 and ert-16, in locus *l* and *o* respectively. Ert-16 is in other respects characteristic — earliness distinctly increased, awns dark-red at the time of maturity — but the density of the spike has remained unchanged, although the base kernels are often protruding as is the case in typical erectoid mutants.

Erectoids Nos. 1—32 were isolated by GUSTAFSSON, ert 33—39 by Dr. J. MAC KEY, ert 40—90 partially by GUSTAFSSON and Dr. N. NYBOM, chiefly by Mrs. U. LUNDQVIST. In the case of *ert-a*^{36} and *a*^{38}, these probably refer to one single mutational event since they arose in the same plant and spike progeny, *i. e.* they are, we consider, identical, not just allelic. Similarly *ert-m*^{34} and *m*^{35} arose in the same plant progeny, but in different spike progenies. In this case the analysis cannot yet decide whether they are caused by one or two separate events. *ert-d*^{30} consists of two different allelic conditions arisen simultaneously. Since the striking differences in phenotype are really due to alleles of the same locus, they are here catalogued as separate events.

Some remarks may be added concerning the origin of the mutations

TABLE 2. *Mode of origin of erectoid mutations in relation to gene locus.*

(Materials from Golden, Maja and Bonus barley)

Locus	γ-rays (acute)	γ-rays (chronic)	X-rays	Protons	Neutrons	α-rays	Sum of mutants	With translocations
a	1	—	10 (11)	—	—	—	11 (12)	—
b	—	—	4	—	—	—	4	—
c	1	1	4	1	7	—	14	at least 6
d	—	1	6	—	2	—	9	1
e	—	—	1	1	—	—	2	—
g	—	—	2	—	2	—	4	—
i	—	—	1	—	1	—	2	—
m	—	—	3	—	4	—	7	—
q	—	—	—	—	1	1	2	—
r	—	—	1	—	1	—	2	—
one-mutant loci	—	—	11	—	1	—	12	—
Sum	2	2	43 (44)	2	19	1	*69 (70)*	*at least 7*
		47 (48)			*22*			

ert-a[77], *c*[75], *c*[85], *c*[68], *d*[89], *e*[65]. The mutation *a*[77] arose after acute γ-irradiation of dormant seeds (16000 r), *ert-c*[75] after γ-irradiation of dormant seeds with 10 r/hour (16000 r), *c*[85] after irradiation of seeds with accelerated protons (showing at the same time sparse and dense ionization effects, EHRENBERG and ANDERSSON, 1954), *c*[68] after chronic irradiation of growing plants for the period of flowering to maturity (*cf.* NYBOM *et al.*, 1956), *ert-d*[89] after chronic irradiation during the entire vegetation cycle, *ert-e*[65] after irradiation of dormant seeds with protons (*cf. ert-c*[85] above).

When analysing Tables 1—3 in some detail we find definite regularities. The most interesting ones are the following:

(1) Mutations of locus *b* have arisen four times, all of them in Golden barley (HAGBERG, 1958; BORG *et al.*, 1958). For reasons mentioned in the papers cited this indicates a genotypical, a variety control of mutation in locus *b*.

(2) 32 per cent of all erectoid mutations, localized so far, have arisen with fast or thermal neutrons, radon α-rays or accelerated protons. All locus-*a*-mutants have arisen with acute gamma- or X-irradiations. Weighing the total number of X- and γ-induced *a*-erectoids against sparse and dense ionization mutants in other loci (including locus *b*), there is a real difference in behaviour (P slightly larger than 0.01**;

TABLE 3. *Mode of origin of erectoid mutations in relation to gene locus.*
(Material from Bonus barley exclusively)

Locus	γ-rays (acute)	γ-rays (chronic)	X-rays	Protons	Neutrons	α-rays	Sum of mutants	With translocation
a	1	—	7 (8)	—	—	—	8 (9)	—
c	1	1	2	1	7	—	12	at least 4
d	—	1	1	—	2	—	4	—
e	—	—	1	1	—	—	2	—
g	—	—	2	—	2	—	4	—
i	—	—	1	—	1	—	2	—
m	—	—	3	—	4	—	7	—
q	—	—	—	—	1	1	2	—
r	—	—	1	—	1	—	2	—
one-mutant loci	—	—	9	—	1	—	10	—
Sum	2	2	27 (28)	2	19	1	*53 (54)*	*at least 4*
		31 (32)			*22*			

excluding locus-*b*-mutants the P-value is exactly 0.01**). Here, as elsewhere, the absolute method of calculating P-values for 2×2-contingencies has been used (KENDALL, 1945, p. 304).

When contrasting the eight X- and γ-mutants in locus *a* of Bonus barley against the 23 sparse and the 22 dense ionization erectoids of other loci, we similarly find a contrast giving a P-value of 0.001***.

Thus, with regard to sparse and dense ionization effects, locus *a* distinctly deviates from the average of other loci.

(3) In the entire material of 69 localized erectoids, there exist at least seven translocations, which have one breakage point at or close to an erectoid locus. One of these translocations occurs at locus *d* (erectoides 7, HAGBERG and TJIO, 1950). All other six translocations have one breakage point at locus *c* (no cross overs have been found). This breakage point lies in chromosome 3 (HAGBERG, unpubl.). Consequently, if we exclude *ert*-c^{82}, c^{83}, c^{85}, c^{88} which have not been analysed so far with regard to chromosome translocation, six out of 10 *c*-mutants have arisen in connection with identified chromosome breakage at locus *c* itself.

The contrast with regard to chromosome translocation is indeed very striking (the P-value for the difference is 1.7×10^{-5}). It shows that locus *c* possesses some distinct properties, not found connected with other loci, especially not with locus *a* and *m*, two of the most frequently mutating loci in addition to locus *c*. We may possibly conclude that

erectoid mutations of most loci arise in consequence of intragenic or »point» changes, whereas locus-*c*-mutations require some sort of chromosome breakage for their origin or, at least, such a pronounced ionization effect at the locus itself that chromosome breakage often results simultaneously with the *ert-c*-mutation.

Such a conclusion is strengthened by the effect of dense ionizations on locus *c*. We find the following conditions:

	Gamma-rays	X-rays	Protons	Neutrons	
With translocation	1	2	—	3	= 6
Without translocation	—	2	—	2	= 4
Translocations not yet analysed	1	—	1	2	= 4
Sum	2	4	1	7	= 14

Eight of 14 cases have originated after proton or neutron treatment. When contrasting the behaviour of the *c*-locus to that of the *a*-locus, we find a difference, giving a P-value of 0.003**(*). The two most frequently mutating erectoid loci thus have different modes of mutation. Locus *a* has so far not been found to mutate with densely ionizing radiations; neither are mutations of this locus connected with chromosome breakage. At locus *c*, on the other hand, dense ionizations favour mutation, and simultaneously chromosome breakage often appears.

(4) The occurrence in numerous other loci of neutron and *a*-ray induced ert-mutations, free from detectable chromosome breakage, supports the view that locus *c* possesses specific properties. At locus *m* four out of seven mutations were induced by neutrons. None, however, was associated with chromosome translocation. At locus *q* both isolated erectoid mutants originated in neutron and radon series. None showed translocation. So an essential point in the origin of locus-*c*-erectoids is the chromosome breakage close to or at locus *c* itself. The high incidence of neutron induced *c*-erectoids is therefore a consequence of the, relatively seen, higher capacity of neutrons to break the chromosomes. What happened in the case of *c*-erectoids, where no detectable chromosome breakage occurs, cannot possibly be judged so far. Offhand, the occurrence of deletions or small inversions cannot be excluded. An interesting point, described by BURNHAM and HAGBERG (1956) as well as HAGBERG (1958), is the simultaneous breakage of a special other chromosome in at least three cases, *viz.* chromosome 2 (*ert-c*^{1}, *c*^{14}, *c*^{47}). However, the three points of breakage in chromosome 2 are not identical.

(5) Conversely, the absence of neutron induced *a*-erectoids can

possibly — by way of guess-work — be interpreted in such a way that owing to their high ionization density neutrons destroy locus *a*, either locus *a* alone or its neighbouring genes, too, and that this destruction of the locus-*a*-region causes an irreparable lethal change. In consequence, neutron induced *ert-a*-mutations would not, or rarely, be realized.

(6) Of 69 analysed erectoid cases, covering 22 different loci, two mutations have shown themselves to be dominant, so that the cross of erectoid times original strain, or that of erectoid times erectoids of other loci, gives an erectoid F_1. Both these cases concern locus *r* (*Ert-r*^{52}, induced by neutrons, and *Ert-r*^{67}, induced by X-rays). In all other instances, the erectoid mutations behave with regard to spike density like recessives or semirecessives of different strength. Whether this dominance is restricted to locus *r* alone and whether all mutations of locus *r* will show such a complete dominance, has to be analysed in further studies of erectoid mutations. There is another difference in behaviour, too, *viz.* that, contrary to the case of erectoids in other loci, those of locus *r* do not react to a treatment with gibberellic acid (STOY and HAGBERG, 1958).

Summarizing the data we conclude, that the three erectoid loci, first denoted in barley: locus *a*, *b*, *c*, have distinct properties of mutability. Locus *a* has so far not given mutations with neutrons. Locus *b* has mutated in just one special variety, not in others. Locus *c*-mutants are associated with chromosome breakage and frequently arise after neutron irradiation. Of other loci, locus *r* produces dominant mutations, with X-rays as well as with neutrons, and locus *d* and *m* show a high incidence of neutron induced mutations but these arise independently of chromosome breakage.

The results indicate how significant is a parallel analysis of gene mutation, using different kinds of ionizing radiations, and that a conclusion from the behaviour of one or a few loci to that of other loci is not correct. This is illustrated by the five erectoid loci *a*, *b*, *c*, *m* and *r*. which all have definite properties or mechanisms of mutation. Furthermore, the results show that different types of ionizing radiations really lead to different results also in a genetical sense.

In the last few years a great number of erectoid mutations have been produced by means of chemical mutagens (ethylene oxide and ethylene imine, EHRENBERG *et al.*, 1959). For instance, in 1958 seven erectoids were isolated in X-rays series, seven in neutron series, five with ethylene

oxide and 19 with ethylene imine. The analysis of these chemically induced erectoid mutants will certainly contribute to a better understanding of the properties of this singular type of mutation.

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HOLOCENTRIC CHROMOSOMES IN ELEOCHARIS

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I. INTRODUCTION

THE behaviour of chromosomes at root mitosis and pollen mitosis of 19-chromosome *Eleocharis palustris* (HÅKANSSON, 1954) and root mitosis of *E. uniglumis* (BATTAGLIA, 1954) has made it very probable that these chromosomes have diffuse or polycentric centromeres, that they are, to use a term of SCHRADER, holokinetic. Such chromosomes have previously been found in *Luzula*. Investigations of x-rayed meiosis and pollen mitosis in *E. palustris* showed that new fragments behaved as new chromosomes taking their place in the metaphase plate. The behaviour at anaphase, however, needed more study. I have now completed the x-ray investigation by a closer study of anaphase and an investigation of *Eleocharis* forms with a lower chromosome number, $n=8$. Thanks to the kindness of Fil. Mag. SVEN-OLOV STRANHDEDE, Institute of Systematic Botany in Lund, I have been able to investigate plants of *E. palustris microcarpa* and *E. mamillata* which have this low chromosome number. STRANDHEDE has collected and determined the chromosome number of a large material of *Eleocharis* subseries *Palustres* (STRANDHEDE, 1958), including the plants investigated here.

Whole spikelets were fixed 24, 72 or 96 hours after x-raying with 500 or 750 r. The slides were stained with crystal violet, a few with Feulgen and after-stained with Lichtgrün.

II. INVESTIGATIONS

Only the observations on pollen mitosis are published, this communication being a supplement to the earlier paper. In seven plants useful divisions were found, one *palustris* plant with $n=19$, two *palustris* with 8 chromosomes and four *mamillata* also with 8. Size differences in the metaphase of pollen mitosis are striking. In the first-mentioned species the chromosome complement approximately consisted of 3 L + 5 M + 11 S chromosomes, but not all L chromsomes showed the same size. In *pal.*

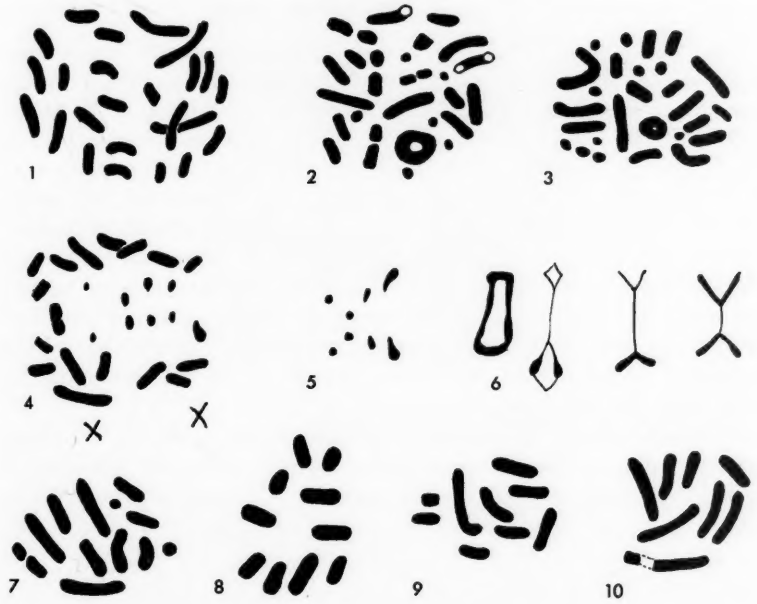
microcarpa the differences are less striking but 2 L+4 M+2 S were sometimes distinct, L having different sizes. In *mamillata* differences were not analyzed; they seemed to represent a similar range.

E. palustris, 19 chromosomes

The investigated plant, irradiated with 500 r, 24 hours earlier, had been collected originally in a locality (Skanör Ljung) where often slightly more chromosomes than 19 are observed (STRANDHEDE, 1958). This may in part explain the fact that in the irradiated pollen cells there was a very strong variation, an unchanged chromosome number hardly ever being observed. This variation was mainly caused by breakage. Strictly di-, tri- or tetraploid numbers did not occur. The new elements may be designated fragments in cases where their diameter is less than that of a normal chromosome, chromosomes if they have about the ordinary diameter. In reality this distinction is only a question of size; a functional difference hardly exists. The highest number of fragments in the plate was eleven, many plates lacking fragments. The chromosome number — the sum of chromosomes and new fragment chromosomes — was often only moderately increased, ranging from 20 to 23, but often considerably higher numbers (28 to 34) were observed. Intermediate numbers, of course, also occurred (Fig. 1). More rarely chromosomes were found which had suffered larger structural changes. Ring chromosomes have been observed (Figs. 2 and 3) whereas other changes are not visible until anaphase. A very long chromosome, rarely observed, was probably an indication of fusion.

The division of fragments in the equatorial plate and a regular separation of the halves to the poles was clearly observed in a number of pollen cells. The fragments were delayed in the plate and divided somewhat later than the chromosomes (Figs. 4, 5). Surely there is a tendency for the fragments to lag and divide later. A certain stickiness, sometimes shown just as the halves separate, seems to be more frequent than in the large chromosomes. This stickiness is doubtless caused by the x-raying, but in this plant it was not very pronounced. In Fig. 4 also very small fragments divide, the division being strikingly similar to a first anaphase of a grass hybrid with bivalent and univalent chromosomes, the latter lagging and dividing in the plate.

The passing of an undivided fragment to the pole also occurs. Fairly frequently a fragment is observed at a varying distance from the pole, but at telophase only very exceptionally is any lagging element notice-



Figs. 1—6: *Eleocharis palustris*, 19 chromosomes. Figs. 7—10: *E. palustris*, 8 chromosomes. — Fig. 1, metaphase plate from pollen mitosis, 24 chromosomes. Fig. 2, plate with ring chromosome and many fragments. Fig. 3, ring chromosome and fragments. Fig. 4, (compare photomicrograph 4 a) anaphase, lagging fragments dividing regularly, one lagging chromosome, all chromosomes at the poles are not drawn. Fig. 5, anaphase, only the lagging, dividing fragments have been drawn. Fig. 6, peculiar bridges at early telophase. Fig. 7, 10 chromosomes, 3 fragments. Fig. 8, 10 chromosomes. Fig. 9, 9 chromosomes. Fig. 10, 8 chromosomes, one with a lacuna.

able; these must have been included in the nuclei. In reality, anaphase was regular in most cells, and it therefore seems that non-lagging fragments divide, probably rather frequently. Lagging of chromosomes was also observed though more rarely (Fig. 4). Peculiar is the lagging of one of the largest chromosomes in the longitudinal direction of the spindle, as already described in an earlier paper.

Stickiness bridges at early anaphase are rare here, but sometimes more permanent bridges are found, some of them indicating structural changes (Fig. 6). Ring chromosomes are expected to form peculiar bridges. Usually, there was only one bridge, very rarely two. In a few cases a double bridge was observed.

E. palustris, n=8

Two plants have been investigated. Fixation made 24 hours after 750 r sometimes showed a very disturbed pollen mitosis. The chromosome number was often high; for instance, in a counting of 13 pollen cells only two had the unchanged number 8, whereas seven had 16 or more. Thus polyploidy was frequent here; one or a few fragments may be present (Fig. 7). Early anaphase shows occasionally one but mostly several bridges, which later disrupt and contract into the telophase groups. This is evident from the fact that in 19 cells with bridges all were in early or middle anaphase, whereas of 19 cells lacking bridges all except one represented near telophase or telophase. This proves that the bridges were typical stickiness bridges. Another anther showed less disturbed pollen mitosis. Increased number of chromosomes but no polyploidy was observed here (Fig. 8).

In fixations made 72 hours after x-raying several spikelets had pollen mitosis. Here conditions were rather regular, the great majority of metaphase plates showing 8 chromosomes. A change of number may occur, 9 or very rarely 10 chromosomes being observed. The higher number was mostly not caused by breakage, changes of size being rarely indicated. However breakage may occur; two small chromosomes lying together were a clear indication of such a process (Fig. 11, compare Fig. 23), but small new chromosomes may also be widely apart. Also, though rarely, very small chromosomes or fragments may be observed (Fig. 11, 12). A lacuna in a chromosome was observed once (Fig. 10). A plate with only 7 chromosomes, one very large, is shown in Fig. 13. Anaphase was very regular here, bridge formation not being observed and lagging being very rare. The behaviour of smaller fragments at anaphase could not be observed as they were too rare.

For the study of pollen mitosis after 96 hours and 750 r only little material was available. The chromosome number could be changed. Fig. 15 *a* and *b* shows the two anaphase plates in a cell with eleven chromosomes. Fragments were hardly observed but this may depend on an insufficient material being investigated; in any case they must be rare. Observed anaphase stages were regular though longitudinal lagging of a large chromosome was once observed.

E. mamillata

Only one plant treated with 750 r and fixed after 24 hours was closely investigated; from three other plants a more incomplete material

Fig. 4 a. First anaphase of meiosis with lagging and dividing fragments. This figure represents the same cell as in Fig. 4, but only the fragments indicated by an x are visible.

was procured. Pollen mitosis in the first-mentioned plant may be described first. Metaphase showed a rather varying degree of disturbance. There was one spikelet with a metaphase variation that recalled the plant with 19 chromosomes first described. In one spikelet the normal number was more often seen but higher numbers were frequent. Fragments were also frequent (Fig. 16). Small dot-like fragments were noted (Fig. 17); they were present in a few pollen cells. Polyploidy caused by fusion of tetrad nuclei was very rare here. Anaphase was on the whole rather regular but lagging and bridge formation occurred not infrequently. Lagging small fragments and fragment chromosomes were observed when dividing. In Fig. 18 such a fragment has remained at the equator. Ordinary chromosomes may also lag but as a rule they are later on included in the nuclei.

Feulgen-staining showed that only very rarely does any chromosome material remain outside the newly formed nuclei. In certain cases two, more rarely one, crystal violet stained bodies were regularly observed and these looked like eliminated chromosomes or micronuclei. Their size was constant, and the form varied, often tapering at one end, more often as a short cylinder. Lichtgrün staining showed their nature of nucleoli or pre-nucleoli (Fig. 19). A third body, observed only exceptionally, was much smaller. These bodies appear at late anaphase or mostly telophase but disappear later. They recall the extra-nuclear bodies seen in meiotic interphase, the latter, however, being spherical

(HÅKANSSON, 1954). The bodies disappear after some time, then a very large nucleolus is formed in the so-called tube nucleus. Sometimes the two bodies were close together as if a division had occurred (Fig. 20), but, usually they are apart. They have therefore probably been formed independently and seem to disappear suddenly. Their substances are probably transported to the tube nucleus.

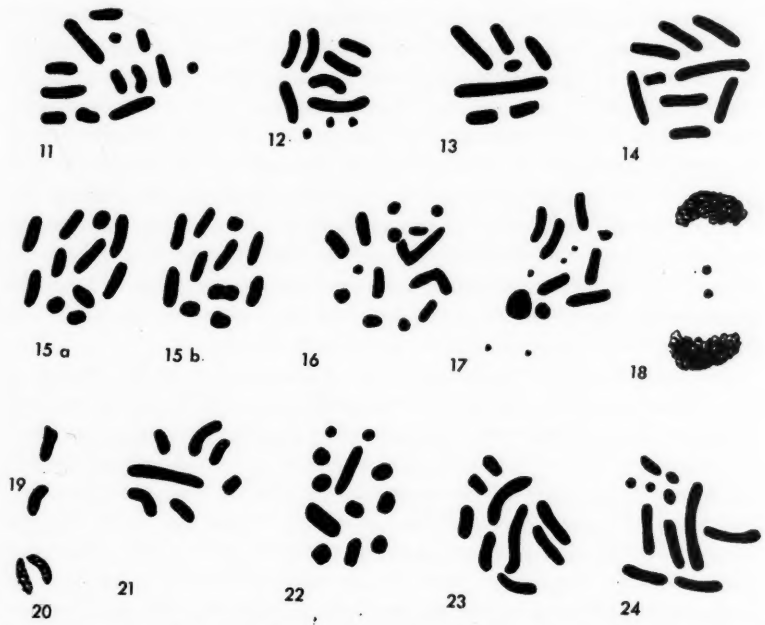
In other investigated *mamillata* plants no good anaphase stages were observed. Metaphase showed rather different frequencies of irregularities. One plant (500 r, 24 hours) had a high frequency of polyploid, especially triploid plates, but also plates with a high, aneuploid number and several fragments. Another plant (750 r, 72 hours) had plates with lower numbers, very often 8; Fig. 21 shows 7 chromosomes, one very long. Fragments were observed (Fig. 22). In this plants there were also some polyploid plates; in such cells the number of aborted tetrad nuclei is less than three. Observed cases of anaphase were few, all were regular. A further plant (500 r, 72 hours) occasionally showed fragmentation of chromosomes (Fig. 23, 24), high numbers were hardly ever observed.

III. CONCLUSIONS

Chromosome changes at pollen mitosis caused by x-rays are visible at prophase but more easily observed at metaphase, all new elements being arranged in the plate. The nature and frequency of changes were very different. The higher dosage 750 r caused more profound changes, and fixations made 24 hours after x-raying showed more disturbances than after 72 hours. This corresponds to expectation.

The typical x-ray effect was breakage, but other changes were also observed. Polyploid plates may be frequent, and as stated in the earlier paper are the result of a union of several tetrad nuclei. Triploid plates may be surprisingly frequent, the union presumably occurring at, or after, the second meiotic division. Observations on meiosis support this view. Irradiation of an anther at this stage may cause a high percentage of polyploidy.

Aneuploid chromosome numbers were often not caused by breakage, the chromosomes not having undergone changes in size. Also in untreated material a changed number of chromosomes in the pollen cells has been occasionally found in *Eleocharis* (*cf.* HÅKANSSON, 1954, p. 243), and numerous instances have been observed by STRANDHEDE (1958) in different *Eleocharis Palustres*. After x-raying the frequency of changed numbers is greatly increased. This variation might be caused by meiotic



Figs. 11—15: *E. palustris*, 8 chromosomes. Figs. 16—24: *E. mamillata*, 8 chromosomes. — Fig. 11, 11 chromosomes, 2 fragments. Fig. 12, 7 chromosomes, 3 fragments. Fig. 13, 7 chromosomes, one very long. Fig. 14, 9 chromosomes, 2 L+5 M+2 S. Fig. 15 a and b, anaphase, each plate having 11 chromosomes. Fig. 16, 8 chromosomes, 6 fragments. Fig. 17, dot fragments in the plate. Fig. 18, telophase, a divided fragment at the equator. Figs. 19—20, nucleolar bodies in the cytoplasm. Fig. 21, 7 chromosomes, one very large. Fig. 22, one very thick chromosome. Fig. 23, 7 normal and 2 fragment chromosomes. Fig. 24, 6 chromosomes, 1 fragment chromosome, 4 fragments.

irregularities, but is this explanation also valid in our case? It is certainly a fact that in *Cyperaceae* — as in *Juncaceae* — the time between meiosis and pollen mitosis is shorter than in other angiosperms, but as stated earlier, the variation already after 24 hours is peculiar. An alternative explanation could be reproduction of one or more chromosomes at prophase. The occasional occurrence of a very thick chromosome speaks in favour of this (Fig. 22) and of, as it seems, paired chromosomes (Fig. 1), but such a cause of aneuploidy in the pollen cell is by no means proved. Rarely were there only seven chromosomes.

Besides chromosomes changed through breakage, structural changes

were in some cases represented by instances of fusion and of ring chromosomes. Other instances were only indicated through peculiar bridges at anaphase. Owing to simple breakage fragment chromosomes and fragments had arisen; they are of very different size as is clear from the previous chapter. Several small fragments of equal size were, however, often observed in the plate; this is shown in several figures indicating that certain fragments of, for instance, the usual satellite size are formed relatively often; they are further observed in many different plates. On the other hand the still smaller dot-like fragments were rarely formed but could occur in a considerable number in the same plate. Small chromosomes and fragments are always in the plate, very rarely are fragments only in the vicinity of the plate.

The behaviour of fragments at anaphase can only be studied in fixations where they are more frequently formed. This has been observed in three plants, one from each species or subspecies investigated. The best divisions were seen in *palustris* with $n=19$. A regular division of fragments of different size, even in very small ones, was observed, the halves going to the poles. The fragments were lagging at the equator, their division being somewhat delayed. They are, however, also able to divide without any delay, but this is more difficult to observe. An undivided fragment may go to the pole: a fragment may be observed at different distances from the pole, but at telophase any fragment on the phragmoplast can be very rarely detected. It is perhaps uncertain whether this should be interpreted as a proof of centric activity of the fragment; in living material of a plant with monocentric chromosomes the transport of a lagging acentric fragment to the pole has been shown (BAJER). A certain cleaning of the phragmoplast from lagging elements seems generally to occur before the forming of nuclear membranes. The behaviour of dot-large chromosomes has not been observed. Not only fragments but also chromosomes may lag at the equator, though more rarely.

At early anaphase there may be a transient bridge formation due to stickiness caused by x-rays. Certain more permanent cases of bridge formation may find their explanation in breakage and sister reunion. These are common types of bridge formation, often observed in x-rayed material. Other bridges are more rare and indicate other structural changes, for instance, a ring chromosome.

The behaviour of the fragments at anaphase must be due to centromeric properties; they are invariably arranged in the metaphase plate, and at anaphase they divide and pass to the poles like ordinary chro-

mosomes. As very small fragments divide, it seems that the *Eleocharis* chromosomes have diffuse centromeres, or if polycentric, that the different centromeres must lie very close. In *Luzula purpurea* x-raying resulted in dividing fragments, except the small fragments, which were therefore interpreted by the Portuguese authors (DE CASTRO *et al.*, 1949) as being only matrix fragments. The same authors are of the opinion that the *Luzula*-chromosomes have »diffuse centromere». On the other hand LA COUR also made x-ray investigations on *L. purpurea* and considers the eliminated fragments as acentric. He concludes that »evidence from x-ray treatment seems to favor a polycentric rather than a diffuse centromere in this species». Unfortunately no evidence of the behaviour of dot-like fragments could be obtained in *Eleocharis*, which would have been of interest in this connection, for to judge from the figures they must be considerably smaller than the eliminated fragments in *Luzula*. The latters are apparently of a size that ought to be followed by division at anaphase in *Eleocharis*.

However, it would be surprising if the centromere structure were very different in *Luzula* and *Eleocharis*, which are the only angiosperms genera having these peculiar chromosomes. It is very probable that they occur in the whole family *Juncaceae* resp. *Cyperaceae*. Some recent authors have stressed similarities between these families indicating a relationship; a connection of *Cyperaceae* with the grass family should not exist. The cytological properties of the latter two families are very different. The peculiarities at mitosis, which have been interpreted as proving holocentric chromosomes in these cases, are absence of centromere constrictions, an unusual stickiness of the matrix and the behaviour of the half-chromosomes at anaphase. They separate parallelly and often the ends are bent towards the pole thus giving the anaphase chromosome a concave appearance.

In still another plant group, the algae Conjugales there are perhaps holocentric chromosomes, namely in the Desmids (KING, 1953) and in *Spirogyra* (GODWARD, 1954). In the latter genus irradiated material has been investigated; large chromosomes were divided into numerous fragments which move to the poles. GODWARD, however, is of the opinion that the large *Spirogyra* chromosomes are polycentric.

REVELL (1953) has shown that sensibility to breakage is low at the beginning, high at the end of the resting stage. This may explain the more regular mitosis in the pollen cell after 72 hours.

SUMMARY

Pollen mitosis was studied in x-rayed spikelets of *Eleocharis palustris* $n=19$ and $n=8$, and in *L. mamillata* $n=8$.

Metaphase showed a number of changes, such as an increased or, rarely, a diminished number of chromosomes, structural changes and fragments of different size.

Anaphase may show lagging and bridges but the most important phenomenon was the behaviour of fragments. They behaved as ordinary chromosomes, in that they divided — often somewhat delayed — in the equatorial plate, the halves going to different poles.

The holocentric nature of the *Eleocharis* chromosomes (diffuse centromere) thus seems to be proved.

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CHROMOSOME STUDIES ON FINNISH PTERIDOPHYTA I

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I. INTRODUCTION

WHEN the lists of plant chromosome numbers were published by A. & D. LÖVE (1948) and TISCHLER (1950), the chromosome numbers of many Pteridophyta were uncertain or wholly unknown. During the past eight years, however, some very important contributions to pteridophyte cytology have been made, especially in England. The best known is, no doubt, MANTON's large work (1950). In her book are given the exact chromosome numbers of many new and also earlier studied species of Pteridophyta collected in Britain and western Europe. In eastern North America the ferns have been investigated cytologically by BRITTON (1953) and WALKER (1953). WALKER (1955) has since studied the *Dryopteris spinulosa* complex in Britain also.

In Finland, there are about 58 pteridophyte species, about half of which are common. None of the Pteridophytes had been studied cytologically in Finland, although most of the species found in Finland had already been investigated in the other countries of Europe. These investigations have been carried out in order to add to our knowledge of the chromosome conditions in the Finnish Pteridophyta. In later publications other problems of pteridophyte cytology will be considered, too.

II. MATERIAL AND METHODS

All the specimens studied during this work were collected in the summers of 1956 and 1957 in the neighbourhood of Helsinki. Preparations were made of different stages of meiosis in the SMCs of about twenty of the commonest pteridophyte species found in this region of south Finland. Fixation was always carried out immediately at the site of growth. Acetic alcohol (1 : 3) only was used for fixing, because chromic and osmic acids in the fixing solution usually coloured the cell walls,

thus hindering the inspection of the chromosomes. The sporangia were kept in acetic alcohol for about 1—4 hours before the fixative was poured off. For staining, acetocarmine was used in most cases. The objects were kept in the staining solution about 24 hours and then transferred to a slide in a drop of 45 % acetic acid. Squashing was carried out with a kind of »foot press». In certain cases the Feulgen stain was also used. The permanent preparations were made with Euparal mounting medium. Microscopying and the camera lucida drawings for figures (1—10) were done with a Zeiss Winkel microscope with 100× apochromatic oil immersion objective (A=1.32) and 16×KPl oculars. The original magnification of the figures was thus about 2500×. Wild KGM 20 microscope with phase contrast optics was also used for examining some difficult objects.

For the nomenclature of the pteridophyte species studied the names recommended by HIITONEN (1933) will be used, but the names used by MANTON (1950) will also be mentioned, if they are different.

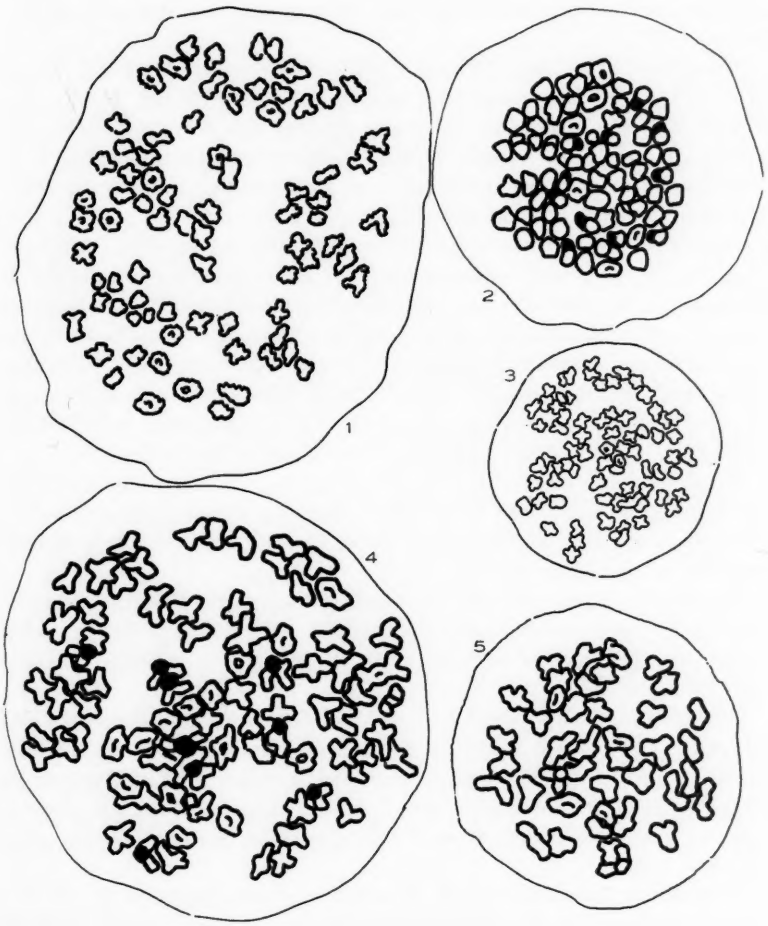
III. OBSERVATIONS AND CONCLUSIONS

L y c o p o d i a c e a e: *Lycopodium inundatum* L. (Fig. 1) is a rather rare clubmoss species in the region of Helsinki, Finland. The chromosome number is obviously the same ($n=78$) as in those specimens studied by MANTON (1950) from Scotland. The shape of the chromosome bivalents differs, however. The bivalent photographed by MANTON (1950) seem to be at the beginning of the first anaphase stage.

In Finland, the commoner *Lycopodium* species *L. clavatum* L. and *L. annotinum* L. also seem to have the same degree of polyploidy as was observed by MANTON (1950) in the British material of these clubmosses.

P o l y p o d i a c e a e: *Polypodium vulgare* L. (Fig. 2). According to MANTON (1947), there are in Britain and the neighbouring parts of the continent of Europe three cytological types of this fern species, diploid, tetraploid and hexaploid, having respectively the gametic chromosome numbers 37, 74 and 111. In Finland, all *P. vulgare* specimens fixed and studied up to now seem to have 74 bivalents at the first meiotic metaphase in the SMCs. Thus the species is tetraploid in Finland. The tetraploid form of *P. vulgare* is the commonest in the western countries of Europe from Scandinavia to the Pyrenees (MANTON, 1950).

Asplenium septentrionale (L.) HOFFM. (Fig. 3). In Finland, this species has 72 bivalents at meiosis. According to MANTON (1950) such



Figs. 1—5. The SMCs of the Pteridophyta during diakinesis and prometaphase I. — Fig. 1. *Lycopodium inundatum* L. $n=78$. Fig. 2. *Polypodium vulgare* L. $n=74$. Fig. 3. *Asplenium septentrionale* (L.) HOFFM. $n=72$. Fig. 4. *Dryopteris spinulosa* (MÜLL.) KTZE $n=82$. Fig. 5. *Dryopteris austriaca* (JACQ.) WOYN. $n=41$. Acetic alcohol+acetocarmine squashes, magnification about $\times 1500$.

a form is to be regarded as tetraploid. The same chromosome number has previously been found for this fern species in Scotland and in Switzerland.

Dryopteris spinulosa (MÜLL.) KTZE=*D. spinulosa* (MÜLL.) WATT (Fig. 4) is quite a common fern species in South Finland. The *D. spinulosa* complex has been especially studied by WALKER (1953 and 1955). *D. spinulosa* seems to have only tetraploid sporophytes in Finland, as also in the other parts of Europe. The number of chromosome bivalents is 82 in meiosis.

Dryopteris austriaca (JACQ.) WOYN.=*D. dilatata* (HOFFM.) A. GRAY (Fig. 5). *D. austriaca* seems usually to have diploid sporophytes in Finland. At the first meiotic metaphase there are only 41 bivalents in this species. Diploid forms of *D. austriaca* have previously been found in Switzerland, Scotland, Madeira, Norway (MANTON, 1950; WALKER, 1955) and also in Germany (DÖPP, 1958). The interesting fern group *D. spinulosa—D. austriaca*, especially the forms with intermediate characters, require more cytological investigation in Finland.

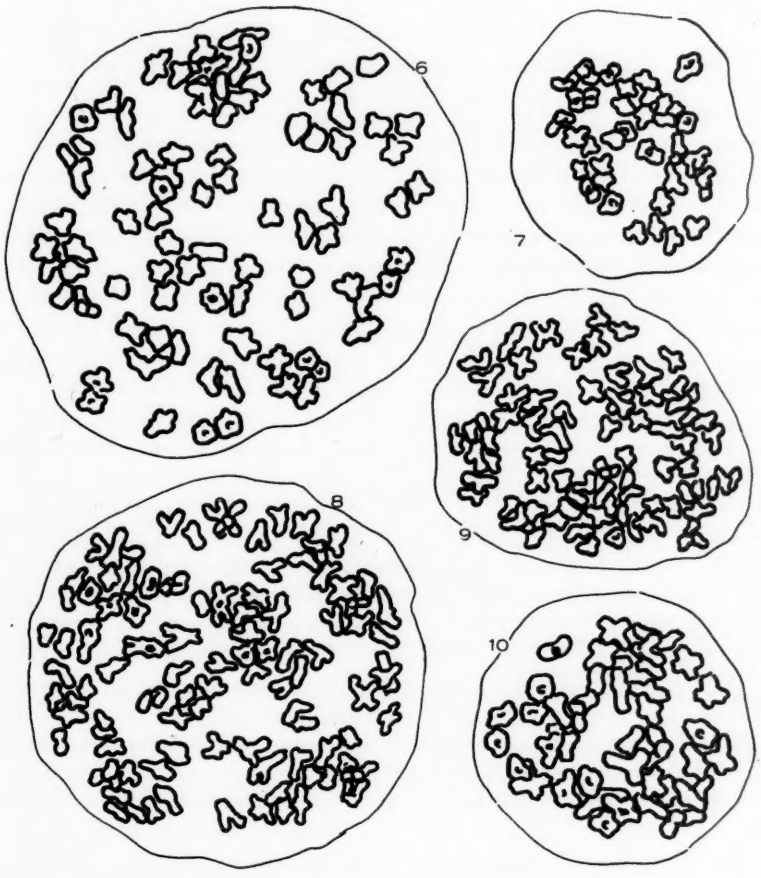
Dryopteris filix-mas (L.) SCHOTT. (Fig. 6) is a very common fern species in South Finland. In its SMCs there are 82 bivalents at meiosis. The same number was reported earlier by MANTON (1950) in British material of the same species.

Dryopteris thelypteris (L.) A. GRAY=*Thelypteris palustris* SCHOTT. (Fig. 7) is obviously diploid in Finland. The gametic chromosome number of this species is 35, which is the same as is found in England (MANTON, 1955).

Dryopteris phegopteris (L.) C. CHR.=*Phegopteris polypodioides* FÉE (Fig. 8). The Finnish form of this species obviously has 90 bivalents at the first meiotic metaphase. The same number was determined by MANTON (1950) from British and Swedish material of *D. phegopteris*, and by BRITTON (1953) from North American material of the same species.

Dryopteris linnaeana C. CHR.=*Gymnocarpium dryopteris* (L.) NEWM. (Fig. 9). The SMCs of the Finnish form of this fern consist of 80 bivalents at meiosis. The same number was counted by MANTON (1950) in British and Swedish material.

Struthiopteris filicastrum ALL. (Fig. 10) has about 40 chromosome bivalents in the SMCs during the first meiotic metaphase. This number of chromosomes was already counted by FRIEBEL (1933) and OKUNO (1936).



Figs. 6—10. The SMCs of the Pteridophyta during diakinesis and prometaphase I. — Fig. 6. *Dryopteris filix-mas* (L.) SCHOTT. $n=82$. Fig. 7. *Dryopteris thelypteris* (L.) A. GRAY $n=35$. Fig. 8. *Dryopteris phegopteris* (L.) C. CHR. $n=90$. Fig. 9. *Dryopteris linnaeana* C. CHR. $n=80$. Fig. 10. *Struthiopteris filicastrum* ALL. $n=40$. Acetic alcohol +acetocarmine squashes, magnification about $\times 1500$.

Acknowledgement. — This study of the cytology of the Finnish Pteridophyta was aided by grants from the Suomen Kulttuurirahasto, for which I acknowledge my gratitude.

SUMMARY

Ten species of the Finnish Pteridophyta, a lycopod and nine ferns, have been investigated cytologically. The chromosome numbers counted in the SMCs of them seem to tally those reported for the same species studied in Europe and eastern North America. Three of the species studied seem to have only diploid sporophytes in Finland, the others are obviously tetraploids.

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SELF-COMPATIBILITY IN TETRAPLOID STRAINS OF TRIFOLIUM HYBRIDUM

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ALSIKE clover (*Trifolium hybridum* L.) was among the first forage crops to which colchicine was applied as an adjunct to breeding, and several tetraploid strains which are equal or slightly superior in yield to adapted diploid varieties have been developed. However, breeding progress with 4N alsike has been relatively unspectacular.

Diploid alsike clover ($2n=16$) is obligately cross-pollinated and seed is obtained only rarely from self-pollinations. Self-incompatibility is due to the action of oppositional S alleles with gametophytic control of pollen reaction (WILLIAMS, 1951; BREWBAKER, 1951, 1953).

Colchicine-induced autotetraploids of alsike clover are often self-incompatible as the diploids. However, fully self-fertile tetraploid sectors were obtained on several self-incompatible diploid plants by BREWBAKER (1953). Self-compatibility was shown to result from the functioning in selfed styles of pollen grains heterogenic for certain S alleles. The S alleles in these grains interacted in such a way (competition interaction) that competition grains were never inhibited in self- or cross-pollinations and functioned in crosses with a frequency comparable to that of other uninhibited grains. Competition interaction similarly has been recognized as the basis for self-compatibility in tetraploids of *Trifolium repens* and *T. pratense* (ATWOOD and BREWBAKER, 1953; PANDEY, 1956).

The apparent selective advantage of competition pollen classes in 4N populations led to the postulate that such classes and the concomitant self-compatibility would monopolize frequencies obtained in 4N breeding populations (BREWBAKER, 1954). The possibility that this self-compatibility might become a significant deterrent to breeding progress with 4N clover provided stimulus for the following study.

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I. MATERIALS AND METHODS

Five tetraploid alsike strains were made available in 1952 through the courtesy of Drs. G. JULÉN (Svalöf, Sweden) and K. FRANDSEN (Ötoftegaard, Denmark). One of these strains (Tetra) has been marketed. Two strains, Sv 0205 and Ötofte 4N, are long-bred but have not been released due to low yields of forage and seed, respectively. Two additional lines (Sv 0211 and Nr 3388) were in the early generations of breeding in 1952. All lines originated from colchicine-doubled plants of self-incompatible 2N varieties.

Approximately 100 plants of each of the five strains were grown to maturity in bee-free greenhouses of the Institute of Genetics, University of Lund, Sweden. From five to ten heads per plant were hand-rubbed on alternate days during anthesis to insure self-pollination. Fewer than five plants per strain were discarded because of poor flowering or indehiscent anthers, and all plants retained for selfing produced abundant stainable pollen.

Four strains were planted in November 1952 and were selfed in the following March and April, while the variety Ötofte 4N was planted in March 1953 and pollinated in mid-June. The low seed sets of Ötofte plants were believed to be attributable in part to excessive heat in the greenhouse during anthesis of this strain. However, this strain has not been marketed primarily because of its poor seed-setting performance.

II. RESULTS

A total of 410 tetraploid plants were pollinated. Seed sets obtained upon selfing ranged as high as 166 per head, while plant averages of the 5 to 10 heads manipulated ranged up to 130.5 selfed seeds (Table 1). Highly self-compatible and self-incompatible plants were obtained in each of the five strains. Continuous variation was observed between

TABLE 1. *Distribution of selfed seed sets of alsike tetraploids.*

	Number of plants in selfed seed set classes of										
Strain	0—2.9	3—5.9	6—9.9	10—19.9	20—29.9	30—39.9	40—49.9	50—59.9	60—69.9	70—79.9	Above 80
Sv 0205	14	6	1	2	7	12	13	13	9	4	9
Tetra	40	16	10	3	3	4	3	4	2	1	1
Nr 3388	38	14	12	8	0	1	2	3	1	1	1
Ötofte 4N	46	11	2	16	7	2	1	0	0	0	0
Sv 0211	41	5	0	5	5	8	3	0	0	0	0
Total	179	52	25	34	22	27	22	20	12	6	11

TABLE 2. *SC* classification of alsike tetraploids.*

4N Strain	2N Parent Lines	Number of Plants SI	: SC*	Percent SC Plants
Sv 0205	Svea×Ötofte 2N	14	76	84
Tetra	Balingsta	40	47	54
Nr 3388	Svea	38	43	53
Ötofte 4N	Ötofte 2N	46	39	46
Sv 0211	Ötofte 2N	41	26	39
Total		179	231	56

these two extremes, the distributions being skewed conspicuously towards low self-compatibility in most strains.

An average of 3.0 seeds per selfed head was selected as an arbitrary dividing-line between self-incompatibility (SI) and self-compatibility (SC). Of the 410 tetraploid plants which were self-pollinated, 56 % were classified as self-compatible (Table 2). Of the 231 SC plants, 154 averaged more than 10 seed per selfed head and can be considered highly self-fertile (Table 1). Frequencies of SC plants in the five strains ranged from 84 % for Sv 0205 to 39 % for Sv 0211. Thirteen plants of Sv 0205 had been selfed in 1951 of which 6 were SI and 7 were SC. Therefore, the totals for Sv 0205 are 20 SI and 83 SC plants, making it significantly more self-compatible than the other strains. This strain was perhaps the most extensive bred of those tested, with the possible exception of Tetra.

It is evident that no matter where the dividing line between SI and SC is drawn the seed sets of plants classified as self-compatible are not distributed normally (Table 1). Considering totals this skewness is

TABLE 3. *Statistics of average and skewness for selfed seed sets of SC alsike tetraploids.*

Strain	Skewness g	t	Average Mean	Median	Number of Plants
Sv 0205	+ 0.16	0.52	48.7	43.8	76
Tetra	+ 1.86	15.47**	23.3	7.2	47
Nr 3388	+ 1.73	4.79**	18.1	7.7	43
Ötofte 4N	+ 1.17	3.09**	14.3	12.8	39
Sv 0211	— 0.37	— 0.82	25.0	27.2	26
Total	+ 1.109	6.93**	29.4	22.1	231

* Plants averaging less than 3.0 seeds per selfed head were considered self-incompatible (SI) and all others, self-compatible (SC).

** Significant at P=.01 level.

evidenced both by the lower median (22.1) and mean (29.4) and by a highly significant t for the »g» test of skewness (Table 3). Selfed seed sets of SC plants in three of the strains were significantly skewed from normality toward values lower than the mean. In lines Sv 0205 and Sv 0211, no departure from normality was detected.

III. DISCUSSION

Self-compatibility (SC) has arisen *de novo* among induced autotetraploids of alsike, white, red and other clovers. In all cases studied, SC results from the predictable behavior of »competition» heterogenic pollen grains. There is little question that competition forms a basis for the observed SC in the *T. hybridum* material considered here. The question immediately arises whether the competition theory can account for the broad and skewed distribution of selfed seed sets in 4N alsike. As will be noted later, such distributions are quite common among partially self-incompatible flowering plants.

The distribution of selfed seed sets in 4N alsike will have been influenced largely by three factors: incompatibility, gametic and zygotic lethality, and general fecundity (*e. g.*, size of inflorescence). Lethality and fecundity are factors of known importance in tetraploid seed production, and of particular significance in inbred populations. While they cannot be ruled out in the interpretation of these data, they are not known to result in the degree of departure from normality noted. As an example, open-pollinated seed sets of 98 relatively unrelated tetraploid alsike plants grown by the author in 1950 were distributed normally, with mean=25.8 and median=24.9.

The influence of incompatibility on the distribution of 4N seed sets, however, is known to be of great significance. This is due to the facts that (a) a high proportion of pollen grains may be inhibited in a genetically fertile mating and (b) the number of seeds obtained in such matings is directly correlated with the percent functional pollen. This correlation was found by ATWOOD and BREWBAKER (1953) to be highly significant for both selfed (r=.962) and crossed (r=.880) seed sets in 4N white clover. According to competition theory self-compatible tetraploids can be expected to fall into seven genotypes having 0, 17, 33, 50, 67, 83 or 100 percent pollen which can function in self-pollinations (chromosome assortment ratios assumed). In studies of white clover (IDEM) plants in the 0 % class were SI, while plants with 50 to 100 % functional pollen set nearly twice as much selfed seed as did plants in the 17 and 33 % classes.

A positive skewness can be conferred on the selfed seed sets of a 4N population, therefore, by a bias in the distribution of the seven incompatibility genotypes. Such a bias is almost inevitable, particularly during early breeding generations. It has been observed that from 5 to 10 % of the S allele combinations in alsike function as competition gametes (BREWBAKER, 1953). This frequency endows approximately 30 to 60 % of the plants in early composite generations with self-compatibility, assuming a fairly broad sample of S alleles among c-doubled parents. A high proportion of these SC plants fall in genotypic classes producing 17 % or 33 % functional pollen. The 83 % and 100 % classes are rare, as they require that competition interaction occur in all three heterogenic gamete classes of a triallelic, or in five or all six of the heterogenic gametes of a tetra-allelic plant. Any increase in inbreeding, through self- and sib-pollination (competition gametes are never inhibited) leads to an increase in the genotypes having 33 %, 50 % and 67 % functional pollen (*e. g.*, genotypes such as S_{1124}, S_{1113} and S_{1122}, assuming S_{12} as a competition class).

The results of an increase in frequency of competition interaction classes in any population, then, would be (1) an increased frequency of self-compatible plants and (2) an increased proportion of SC plants in the higher classes of functional pollen. As a result, the skewness of selfed seed sets might tend to become less pronounced as the proportion of self-compatible plants increased in the population. The variety Sv 0205, in which a significantly high proportion (84 %) of the plants were classified as self-compatible, conforms to this expectation. Whereas the distributions of three other strains, with roughly 50 % SC plants, were significantly skewed to the lower selfed sets, that of Sv 0205 did not differ significantly from normality. In a fifth strain Sv 0211 the comparatively small sample of 26 SC plants was not conspicuously skewed, although the proportion of SC plants was low (39 %).

Selfed seed and fruit sets similar to those observed here, with distributions varying from SI to full SC and often positively skewed, have been observed in a number of species (*e. g.*, *Medicago* spp., *Lotus corniculatus*, *Melilotus* spp., *Allium* spp., *Narcissus* spp., *Caragana arborescens*, *Prunus* spp., *Dactylis glomerata* and *Phleum pratense*). Polyploidy occurs in most of these species and, with the possible exception of the grasses (LUNDQVIST, 1957), all may be predicted to be of a gametophytic S system in which competition interaction can occur if the locus is duplicated (BREWBAKER, 1957). Thus, competition interaction may provide a plausible explanation both for the presence of

self-compatibility and for its variability in these species. Among recent studies of interest is that of CRAM (1955) on the Siberian pea tree (*Caragana arborescens*). Pod sets obtained upon selfing 218 trees ranged continuously from 0 to 100 % and were strongly skewed towards low self-compatibility. Competition theory could be invoked for such a diploid ($2n=16$) species only upon the assumption of intragenomic duplication of the S locus.

Tetraploid alsike clearly presents a very different target for the breeder than does diploid alsike. Whereas 2N alsike plants rarely if ever set selfed seed in the field, the 4N strains sampled in this study could be expected to undergo considerable inbreeding under field conditions. Self-compatible white clover plants are known to set as much as 80 % selfed seed under field conditions (ATWOOD, 1945). The conclusion appears to be warranted that such inbreeding will be sufficient to lead to a significant depression of vigor and yield, despite the buffering effect of the tetraploidy.

Apart from the possible utility of self-compatibility in breeding 4N clover, it is apparent that it might be desirable to eliminate the self-compatibility altogether from breeding strocks. It should be feasible to accomplish this at any stage in the breeding program. Selection of SI 4N plants alone would not eliminate SC from the population, since self-compatibility may arise in the progeny of any two SI parents. However, *no less than* 25 % and as many as 100 % of the progeny of SI×SI crosses can be expected to be SC if new competition classes have occurred. Therefore, self-incompatible varieties could be made up from SI clones whose diallel progenies have proven fully self-incompatible.

SUMMARY

Five breeding strains of tetraploid *Trifolium hybridum* were tested for the presence of self-compatibility. Whereas self-compatible plants are rare in diploid alsike clover, over half of the tetraploids tested (231 out of 410) were classified as self-compatible. Selfed seed sets ranged continuously up to 130 seeds per head, with distributions strongly skewed toward low self-compatibility. The proportion of self-compatible plants in the five lines ranged from 39 to 84 percent.

Self-compatibility has been encountered previously in colchicine-doubled material of alsike, white and red clovers, among other species. In each case it has been based on the competition interaction of S alleles in certain pollen classes. The competition theory and the rela-

tionship of selfed seed sets to percent functional pollen in 4N plants provide a plausible basis for the occurrence of self-compatibility, its variability, and its departure from normality in the five strains of 4N alsike studied.

It is inferred that self-compatibility and the attendant inbreeding may be undesirable for the development of vigorous tetraploid varieties, and a method based on competition theory is outlined for the elimination of self-compatibility from a 4N population.

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BRIEF REPORTS

ARNE MÜNTZING: Sublethal species hybrids in *Potentilla*.

(Received March 29th, 1958)

The group *Collinae* of the genus *Potentilla* is supposed to be of hybrid origin, having arisen by crosses between members of the *Argenteae* and the *Aureae vernales* (ASCHERSON und GRAEBNER, 1900—1905). Crosses between *P. argentea* and, on the other hand, *P. Tabernaemontani* and *Crantzii*, representing the supposed parent groups of the *Collinae*, were undertaken, but only maternal plants and no true hybrids were obtained (MÜNTZING, 1928). This work, however, in combination with cytological observations, revealed that the *Potentilla* species used were all apomictic. This was also found to be true of *P. collina*, which showed the same constancy and inability to form hybrids as the supposed parent species.

Subsequently, new attempts were made to synthesize *P. collina*, but this time with the help of new sexual or partially sexual strains of *P. argentea* and *P. verna*. A partially sexual *argentea* strain, denoted A—C (A. and G. MÜNTZING, 1941, pp. 252—257; 1945) probably corresponds to the South Italian *argentea* variety *calabra* SER. This strain — as the female parent — could be successfully crossed with other biotypes of *argentea* (A. and G. MÜNTZING, 1945; MÜNTZING, 1958). A—C is diploid with $2n=14$, the other (apomictic) *argentea* types used for the crosses being diploid, tetraploid or hexaploid. In the crosses between A—C and hexaploid apomicts most of the hybrids were pentaploid, resulting from fertilization of unreduced embryo-sacs. In the other cross combinations reduced embryo-sacs were fertilized. In addition to true hybrids the crosses also resulted in the formation of a few maternal plants.

In 1944 attempts were made to hybridize A—C and T—A, a hexaploid Swedish biotype of *P. Tabernaemontani* ($2n=42$). Some seeds were obtained, and these were germinated in 1945, giving a total of 17 seedlings. Root tips were fixed and revealed the following chromosome numbers:

$2n$:	14	21	34	35
Number of seedlings:	13	1	1	2

The plants with $2n=14$ and $2n=21$ were all viable and morphologically maternal. The diploids had certainly arisen from pseudogamous development of unreduced ovules. The origin of the single triploid is more difficult to explain. As it was quite maternal and showed no similarity to the other parent, it could only have arisen in one of the following two ways: 1) by the parthenogenetic development of the egg cell in a triploid embryo-sac, 2) by fertilization of a diploid egg cell with a reduced pollen grain of A—C. The latter alternative implies that emasculation of the maternal flower had not been entirely successful.

Of the three precisely or approximately pentaploid plants those with $2n=35$ did not survive transplantation to the experimental field, and for that reason no information concerning their morphology is available. However, the third pentaploid, having $2n=34$, survived transplantation and was observed in the field. This plant had very poor vigour and slow growth. It lived in the field

about a month but died without having flowered. The plant was undoubtedly a true hybrid but only capable of forming leaf rosettes. The leaves were disharmoniously built with rolled up edges and were less hairy than in the mother strain.

Other attempts to synthesize *P. collina* were made with the help of a tetraploid strain of *P. verna* from the Tyrol. This strain is sexual and morphologically variable. Used as the female parent in crosses with Scandinavian apomictic *argentea*-types, it produced no seeds at all. However, when used as male parent in crosses with the A—C strain, some true hybrids were actually formed. Altogether 25 seedlings were raised from crosses between A—C and the *verna* type in question. The following chromosome numbers were found:

Somatic chromosome number:	14	21	28	40	±41
Number of seedlings:	8	1	13	2	1

The individuals with $2n=14$ were all vigorous maternal A—C plants which survived transplantation to the experimental field. In complete contrast to this, all the polyploid plants died young before they could be observed in the field. However, their chromosome numbers demonstrate that all of them were true hybrids. Only one plant, the triploid having $2n=21$, represents the sum of the reduced chromosome numbers of the parents. The twelve plants having $2n=28$ must be the result of unions of unreduced egg cells of A—C with reduced male nuclei of the *verna* parent. The plants with 40 or 41 chromosomes may have arisen from fertilization of egg cells having twice the somatic chromosome number (*cf.* A. and G. MÜNTZING, 1944) or from doubling of the chromosome number in fertilized egg cells having 20 or 21 chromosomes.

From the negative results described above it would appear that hybrids between the *Potentilla* groups *Argenteae* and *Aureae vernales* are generally inviable and that, consequently, *P. collina* cannot have arisen from such crosses. However, such a conclusion would be premature as RUTISHAUSER (1943) succeeded in hybridizing another member of the *Argenteae*, *P. canescens*, as the female parent with a strain of *P. verna*. The single hybrid plant was viable and in several respects morphologically similar to *P. collina*. Thus, the poor vigour of the hybrids described in the present report may be due to the specific genetic constitutions of the parental biotypes. At any rate, the supposed hybrid origin of *P. collina* has not been disproved, and further work, involving other biotypes, may give more positive results.

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GÖSTA ZETTERBERG and NILS FRIES: Spontaneous back-mutations in *Ophiostoma multiannulatum*.

(Received April 17th, 1958)

Biochemical mutant strains of *Neurospora* have been observed to revert back to the wild type (BEADLE and TATUM 1945). Variation in the frequency of such back-mutations has proved to be a useful tool in experiments with physical and chemical mutagens (GILES and LEDERBERG 1948, KÖLMARK and WESTERGAARD 1949). The present authors have performed similar experiments with *Ophiostoma multiannulatum*, chiefly to compare the mutagenic effect of various chemicals, notably purine derivatives. The occurrence of spontaneous back-mutations in mutant strains induced during the last two years by means of the procedures earlier described (FRIES 1948) was first investigated. The results of this phase of the more extensive investigation are reported in this paper.

Methods

Stock cultures of the strains were carried on nutrient agar slants. Bits of mycelium from the slants were used to inoculate liquid »modified medium 3» (FRIES 1949), to which was added the metabolite specifically required by the mutant strain in question. The conidia formed served as inoculum for a second set of similar cultures. These were grown for 1 to 3 days at 30°C on a shaker. Under such circumstances *Ophiostoma* grows in the conidial form. Some strains formed short hyphae in addition to conidia. In such cases the suspension was filtered through sterile cotton. After repeated washing the conidia were resuspended in sterile water to give a density of 2 to 10×10^7 conidia per ml as determined by direct counts in a haematocytometer. The conidia have one nucleus each (personal communication from A. von HOFSTEN).

Twenty plates were prepared to test for back-mutations by mixing 1.0 ml of the conidial suspension with 15 ml of minimal medium (»modified medium 3» less asparagine and inositol) containing 1.5 % agar. The viability of the conidia was determined by inoculating 10 petri dishes containing the minimal medium plus the metabolite required by the mutant strain. The inoculum here was 1 ml of the suspension diluted to contain about 100 conidia per ml. The plates were incubated at 25°C for about 5 days. On the plates with minimal medium only those conidia grow out which have reverted back to the wild type. Such back-mutants form distinct mycelia that can easily be counted. In each experiment from 5 to 10×10^8 conidia were tested. It was concluded that back-mutations occurring with a frequency higher than 10^{-8} should be detected.

Results and Discussion

Of the 93 strains tested, 31 per cent showed back-mutation with a detectable frequency (Table 1). Whether the back-mutations were real or mutations of a suppressor gene has not yet been determined.

In Table 1 the mutant types are arranged with those having the highest per cent of back-mutating strains at the top. The uracil-requiring strains seem to be least stable. Since the inability to synthesize uracil is not caused by identical genetic blocks in these strains (as shown by mating experiments), the instability cannot be due to only one labile gene. The differences in the back-mutation frequencies observed between the different biochemical types of strains may be more apparent than real.

Mutants induced with UV revert more easily to the wild type than those induced with X-rays. Of the 24 strains of X-ray origin only 2 underwent back-mutation spontaneously. These results agree with those obtained by GILES (1956). He tested 23 *Neurospora* mutant strains of X-ray origin for their ability to revert to the wild type. Only 7 of these did so. On the other hand, out of 5 strains induced with UV all showed spontaneous back-mutation. Thus, a qualitative difference between the effects of radiation with UV and X-rays seems to be substantiated. This difference can be satisfactorily explained if it is assumed that UV causes small, usually repairable, defects in the genome, while the destruction by irradiation with X-rays can be more extensive and less readily overcome.

TABLE 1. *Spontaneous back-mutations in mutant strains of Ophiostoma induced with UV or X-rays.*

Mutant type	Induced with	Number of strains tested	Number of strains back-mutating	Per cent of strains back-mutating	
Uracil-less	UV	22	13	59	52
	X-rays	5	1	20	
Methionine-less	UV	21	10	48	33
	X-rays	12	1	8	
Lysine-less	UV	8	2	25	22
	X-rays	1	0	0	
Hypoxanthine-less	UV	11	2	18	13
	X-rays	5	0	0	
Arginine-less	UV	6	0	0	0
	X-rays	1	0	0	
Guanine-less	UV	1	0	0	0
Total number UV-induced strains		69	27	39	31
„ „ X-ray- „ „		24	2	8	

It is a well established fact that the mutation frequency of one gene can be increased by one particular mutagen but not by another while the mutation frequency of another gene may be influenced by the latter mutagen but not by the former. Furthermore, it has been emphasized by KÖLMARK (1957) that a gene can be injured more or less severely, depending on the mutagenic agent used. Recent studies (reported by BEADLE 1957) also indicate a more complicated structure of certain genes than had earlier been assumed. Since gene mutations comprise all changes not visible as chromosomal aberrations it can thus be easily imagined that the structure of one gene may be altered in many different ways, depending on the mutagenic treatment applied. Some of these changes, and perhaps particularly those induced by X-rays, may be so complex that they revert no more easily than do true chromosome aberrations.

The investigation has been aided by grants from the Swedish Research Council of Agriculture.

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W. M. DE BRUYN and E. HANSEN-MELANDER: The chromosomes of the MB lymphosarcoma. A preliminary note.

(Received May 12th, 1958)

In 1947 one of us (DE BRUYN 1949 a) established the MB lymphosarcoma of the mouse (T 86157) (DE BRUYN *et al.* 1949 b) in tissue culture as a permanent cell strain, later known as strain I. From the beginning this culture comprises two morphologic types of cells, mesenchyme cells and lymphoblast-like or round cells. Strain I has retained tumour producing properties which have been referable to the round cells. Several substrains have been derived

from strain I, differing in their cell composition and their capacity to produce tumours.

Thus, in 1948 two new strains were developed, neither of which was capable of producing tumours on injection into susceptible mice. One of these (strain II) contains only fibroblasts and the other (strain III) consists exclusively of lymphoblast-like cells (DE BRUYN 1957).

In 1957, nine MB lines were subjected to chromosome analysis (by HANSEN-MELANDER). Nearly all lines studied differed from each other as well as from normal mouse tissue in their chromosome pattern, each strain having its individual stemline karyotype. Both near-diploid and near-tetraploid chromosome numbers were found.

Eight out of nine strains studied were characterized by a varying number of metacentric chromosomes. Some data are given below concerning strain III, which is especially interesting. The number of chromosomes is hypodiploid, 28—35, 31 on an average, while the number of arms varies from 43—50, averaging 46. It seems that the ratio of telocentrics to metacentrics is correlated with the chromosome number. The varying combinations of telocentrics and metacentrics in cells with the same total number of arms may often be accounted for by assuming a continuously acting instability of the centromeric region, fusions and subsequent transversal separation of arms being frequent. The alternative interpretation would be the coexistence of a great number of relatively stable stemlines with the chromosome arms in different combinations. This point may be studied in clones isolated from this material.

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